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Award Number: DAMD17-97-1-7137

TITLE: The Role of PTHrP in Epithelial Stromal Interactions
During Breast Development

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REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jul 97 - 30 Jun 00)

4. TITLE AND SUBTITLE
The Role of PTHrP in Epithelial Stromal Interactions During Breast Development

5. FUNDING NUMBERS
DAMD17-97-1-7137

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
We have previously shown that PTHrP is necessary for mammary development. Our studies have suggested that PTHrP is involved in regulating epithelial-mesenchymal interactions during both embryonic development as well as ductal outgowth during adolescence. It is our hypothesis that PTHrP is an epithelial signals that modulates the ability of the mammary mesenchyme to support epithelial morphogenesis. Over the three years of this project, we have generated data to support this hypothesis. We have shown that PTHrP, and its receptor PPR1, are expressed in an epithelial-mesenchymal pattern throughout mammary development. In addition, we have demonstrated that PTHrP signaling from the mammary epithelium is necessary for the proper differentiation of the mammary mesenchyme. We have also demonstrated that PTHrP is involved in regulating hormonally-stimulated growth at the terminal endbud during puberty. Finally, using a tetracycline regulated double transgenic mouse we have shown that PTHrP-overexpression before birth casues defects in the branching pattern of the epithelial ducts during puberty, while PTHrP overexpression after birth causes defects in ductal elongation during puberty. These results suggest that PTHrP may have a dual role in mammary development, Before birth, PTHrP may be involved in altering the differentiation of the dense mammary mesenchyme allowing it to support subsequent branching morphogeesis of the epithelium. After birth, PTHrP is involved in regulating cellular proliferation at the terminal end bud during puberty. Together these results support our hypothesis that PTHrP is an epithelial signal that modulates mammary stromal cell function during both embryonic development, as well as adolescent development of the mammary gland

Organogenesis, branching morphogenesis, mammary gland development, transgenic mice, estrogen and progesterone stimulated growth

15. NUMBER OF PAGES
61

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Maureen E. Dunbar 7/25/00

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Introduction

Previous studies from our laboratory have demonstrated that parathyroid hormone-related protein (PTHrP) is necessary for embryonic mammary gland development as well as ductal morphogenesis during adolescence (Dunbar and Wysolmerski, 1999). Overexpression of PTHrP in mammary myoepithelial cells, driven by the keratin-14 (K14) promoter, leads to severe defects in ductular proliferation and branching during puberty, as well as reduced lobuloalveolar development during pregnancy (Wysolmerski et al., 1996). Underexpression of PTHrP or its receptor, PPR1, results in the complete absence of a mammary epithelial duct system and the absence of nipples (Wysolmerski et al., 1998). We have shown that PTHrP is a product of mammary epithelial cells, and that PPR1 is a product of mammary stromal or mesenchymal cells (Dunbar and Wysolmerski, 1999). These studies suggest that PTHrP participates in the regulation of epithelial-mesenchymal interactions during mammary development. In this project, it was our hypothesis that PTHrP is an epithelial signal that acts on mammary stromal cells and modulates their ability to support epithelial morphogenesis. To test this hypothesis, we proposed three technical objectives that were aimed at elucidating the mechanisms by which PTHrP regulates epithelial-stromal interactions in the developing mammary gland. Over the course of this project, we have gathered evidence to support our hypothesis. The following sections report the findings of this project over the three years of funding.

Body

Objective 1: Temporal-spatial expression of PTHrP and PPR1 during mammary gland development

The main goal of this objective was to define the pattern of PTHrP and PPR1 to as PPR1 expression during several stages of mammary gland development. Because the phenotypes of the PTHrP over- and underexpression models suggest that PTHrP is involved in regulating ductal morphogenesis during embryonic development, during puberty and during pregnancy, we concentrated our efforts at analyzing PTHrP and PPR1 expression during these time points. Summarizing this data, we have found by in situ hybridization, that PTHrP is expressed in mammary epithelial cells while PPR1 is expressed in the surrounding stromal cells throughout mammary gland development (see Fig. 2 in *Developmental Biology*, 1998). During embryonic development, PTHrP expression is very intense in the epithelial cells of the embryonic mammary bud, while PPR1 expression is found in mesenchymal cells immediately surrounding PTHrP expressing cells. During puberty, PTHrP expression is found in the epithelial cells of the terminal end bud, specialized structures that serve as the sites of active cellular proliferation and differentiation during puberty, but not in the mature epithelial ducts. PPR1 expression is found in the stromal cells immediately surrounding the TEB. Finally, during pregnancy, there appeared to be a low level of expression of PTHrP in the epithelial cells of the lobuloalveolar units. In addition, PPR1 is expressed at low levels in the fat pad stroma.

Together these results demonstrate that during these stages of mammary development, PTHrP and PPR1 are expressed in an epithelial-mesenchymal (or stomal) pattern, suggesting that they play a role in epithelial-mesenchymal interactions in the mammary gland. Interestingly, the most intense expression of PTHrP and PPR1 are in the embryonic mammary bud and in the terminal end buds during puberty, both sites of active ductal morphogenesis. This suggests that PTHrP and PPR1 may be involved in regulating ductal growth during embryonic development and adolescence. These data are consistent with the phenotypes of the PTHrP under- and overexpression models that show defects in embryonic development and pubertal development, respectively. The results from these experiments have been published in *Developmental Biology* and a re-print of this article has been included in the appendix.

Technical Objective 2: Effects of PTHrP on growth factor production by mammary stromal cells

The central hypothesis underlying this proposal is that PTHrP affects epithelial ductal branching morphogenesis by regulating mammary stromal cell function. Over the past three years we have gathered considerable evidence to support this hypothesis. Several of the experiments discussed in this section

were not part of the original proposal as they followed from data that was unknown at the time of submission. As a result they are not part of the original statement of work. However, we felt that these experiments are related to the overall goal of this project and that the results are important in understanding completely the role of PTHrP in mammary development.

PTHrP changes mesenchymal cell fate decisions

Before submission of this proposal, we discovered that underexpression of PTHrP results in the complete failure of embryonic mammary development. However, the reason for this phenotype was unlcear. As reported in last year's annual report, we now have evidence that the defect in the PTHrP-knockout is due to alterations in the differentiation of the dense mammary mesenchyme. Before discussing this data it detail, it is first useful to review the embryonic development of the mouse mammary gland. A schematic diagram of this process is shown in Fig. 1. In mice, mammary development begin on E10 with the formation of five pairs of mammary buds which are thickenings of the epidermis along an imaginary line, known as the milk line, that extends from the anterior to the posterior limb buds. The mammary buds are fully formed by E12 and are composed of a bell shaped mammary epithelial bud surrounded by a dense mammary mesenchyme.

An important aspect of embryonic mammary development is the sexual dimorphism that occurs after the formation of the mammary bud (see Fig. 1). In female mice, the mammary bud remains quiescent until E16 when they undergo a transition into the second step of embryonic development, the formation of the rudimentary ductal tree (Sakakura, 1987). This process involves the elongation of the mammary bud, its penetration into the mammary fat pad precursor and the initiation of branching morphogenesis. By birth, the mammary gland consists of 15-20 branching epithelial ducts contained within a mammary fat pad. This initial pattern persists until puberty, at which time the mature virgin gland is formed through a second round of branching morphogenesis, regulated by circulating hormones.

In male mice, on E13 the fetal testes begin to produce androgen, which leads to the destruction of the mammary buds (Sakakura, 1987). In response to androgens, the mammary mesenchyme condenses around the neck of the mammary bud, and by E14, severs its connection to the epidermis. The remaining epithelial cells subsequently degenerate by birth. This process has been studied in some detail and it is known to rely on a series of epithelial-mesenchymal interactions . First, androgen receptor expression is induced in the mammary mesenchyme between E12 and E14 under the direction of mammary epithelial cells . The mesenchymal cells are the cells that subsequently respond to fetal androgens, and they in turn sever the epithelial stalk and destroy the epithelial cells . Although the molecular details of this process have not been elucidated, it is clear from the existing literature that epithelial cells and

mesenchymal cells cooperate with one another in the destruction of the mammary bud in response to androgens .

In the absence of PTHrP there are two major defects in embryonic mammary development. As summarized in the original proposal, we found that ablation of the PTHrP gene leads to a failure of mammary development at the transition of the mammary bud into the initial phase of branching morphogenesis. These data have been published in *Development* and a reprint of this manuscript is included in the appendix . In addition to the compete failure of mammary epithelial development in female PTHrP-knockouts, there is also a loss of the normal sexual dimporhism between male and female mice. In examining the mammary buds in PTHrP and PPR1-knockout male embryos at E15, we were suprised to find that the mammary buds do not degenerate on E14-E15 as in the wild type littermates. However, in PTHrP and PPR1 knockout embryos at E15, there is a complete lack of the androgen mediated mesenchymal condensation and the neck of the mammary bud is well preserved (see Fig.1 in *Development,* 1999) As a result, the mammary buds in male and female PTHrP-knockout embryos are indistinguishable at E15. The mutant buds persist until E16-17, at which point they fail to undergo the initial round of branching morphogenesis and instead degenerate, findings identical to that previously reported for female PTHrP- and PPR1-knockout mammary rudiments .

The androgen-mediated destruction of the mammary bud is dependent on the presence of functional androgen receptors in the dense mammary mesenchyme, and the expression of these signals is induced by the mammary epithelium. The absence of the androgen response in the PTHrP- and PPR1-knockout buds combined with the epithelial expression of PTHrP and the mesenchymal expression of PPR1, led us to hypothesize that PTHrP might be the epithelial signal responsible for the induction of androgen receptor expression in the mesenchyme. Indeed, we have shown that there is an absence of androgen receptor expression in PTHrP and PPr1 knockout mice (see Fig. 3 in Development, 1999).

Androgen receptor expression is one of the characteristics of the mammary mesenchyme that sets it apart from the dermal mesenchyme . Therefore the absence of androgen receptor expression in the PTHrP- and PPR1-knockouts suggested that there might be more fundamental defects in the differentiation of these cells. The other classic marker of the specialized mammary mesenchyme is tenascin C . Tenascin C is a heparin sulfate proteoglycan that is present within the extracellular matrix of the condensing mesenchyme associated with several developing organs. Therefore, we also examined tenascin C expression in our PTHrP and PPR1 knockout mice. We found that, similar to androgen receptor, there was no tenascin C expression in the PTHrP and PPR1 knockouts (see Fig.3 in *Development,* 1999), suggesting that the mammary mesenchyme does not differentiate properly in these mice.

These results are interesting on two levels. First, they lend support to our hypothesis that PTHrP contributes to mammary mesenchymal cell function, as

both classical markers of this phenotype are absent in these cells in the absence of PTHrP. Second, these results provide a mechanistic explanation for the failure of the androgen-mediated destruction of the knockout mammary buds. That is, PTHrP appears to be the epithelial signal that induces androgen receptor expression within the mesenchymal cells, and without PTHrP the mesenchymal cells fail to express this receptor and become deaf to the androgen signal. These results have recently been published in *Development* and a copy of this article has been included in the Appendix of this report .

PTHrP signaling through the mammary mesenchyme is necessary for epithelial ductal branching morphogenesis

Another series of experiments we performed to test our hypothesis that the mammary stroma is the critical target for PTHrP's actions in the mammary gland were tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild type and PPR1 knockout embryos. In these experiments, knockout and wild type mammary epithelial buds and mammary mesenchyme were recombined in all possible combinations and grown under the kidney capsule of recipient females. We reasoned that if our hypothesis was valid, and PTHrP and PPR1 do represent an epithelial-mesenchymal signaling circuit, the PPR1-null phenotype would be expected to segregate with mesenchymal tissue. That is, receptor knockout mesenchyme should not be able to support the outgrowth of either receptor knockout or normal epithelial buds, but receptor knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Summarizing the results from these experiments, we found that the PPR1 mesenchyme was unable to support the growth of either normal or PPR1 knockout epithelium (see Fig.7 in *Developmental Biology*, 1998). In contrast, the normal mesenchyme was able to support the ductal outgrowth of both normal and PPR1 knockout epithelium. These results suggest that PPR1 knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. In addition these results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PPR1 knockout mice segregate with mesenchymal tissue and demonstrate conclusively that the mesenchyme is the critical target for the actions of PTHrP during ductal mammary morphogenesis. These results have been published in *Developmental Biology* and a re-print of this article is included in the Apenndix.

Effects of PTHrP on growth factor production inn primary cultures of mouse mammary stromal cells

Now that we have conclusive evidence that the mesenchyme (or stroma) is the target for PTHrP's actions in the mammary gland, our next goal is to determine the molecular mechanisms by which PTHrP exerts its actions. To this end, we proposed two technical objectives that were designed to identify potential downstream signaling partners to PTHrP in primary cultures of mouse

mammary stromal cells. Under technical objective 2, we designed experiments to examine the affects of PTHrP on the expression three candidate factors produced by mammary stromal cells that we felt were likely downstream targets of PTHrP's actions. These three growth factors were hepatocyte growth factor (HGF/SF), insulin-like growth factor (IGF-1), and keratinocyte growth factor (KGF).For these experiments, primary cultures of mouse mammary stromal cells were treated with PTHrP for various times, and growth factor expression was analyzed by either RNase protection analysis or Northern blot analysis. Unfortunately, as mentioned in last years annual report, we were unable to demonstrate any changes in the expression of HGF/SF, KGF or IGF-1 in our culture system. It has recently been reported HGF/SF knockout mice, KGF-knockout mice (12) and IGF-1-knockout mice (13) do not have a mammary phenotype and therefore do not phenocopy the PTHrP-knockout. Based on these findings we feel that it is highly unlikely that these growth factors act downstream of PTHrP during embryonic mammary development, and have decided against compiling a list of other growth factors that may be targets of PTHrP signaling in the mammary gland. Instead, we decided to focus our attention on defining comprehensively the changes in mesenchymal gene expression elicited by PTHrP. These experiments are discussed in detail under technical objective 3.

PTHrP-overexpression during puberty antagonizes the growth promoting effects of estrogen and progesterone

As discussed in the original proposal, characterization of the PTHrP-overexpressing mice (K14-PTHrP mice) demonstrated defects in ductal outgrowth during puberty and early pregnancy, both periods of intense hormonally stimulated growth (Wysolmerski et al., 1996). These results suggested that perhaps PTHrP was involved in regulating hormonally stimulated growth. Consistent with this, we have found that the expression of PTHrP and PPR1 is very intense in the terminal end buds, which are the sites of active cellular proliferation and differentiation of the mammary ducts during adolescence. These findings are interesting because they suggest that PTHrP and PPR1 might be involved in regulating hormonally stimulated growth at the TEB. In our original proposal, we hypothesized that PTHrP might affect the estrogen and progesterone induced production of growth factors in mammary stromal cells that are required for mammary epithelial cell proliferation. We planned to test this hypothesis by examining the expression of HGF/SF, KGF and IGF-1 in mammary stromal cells in response to PTHrP in the presence of estrogen and progesterone. However, we are having difficulty seeing a response in our stromal cell cultures in response to estrogen and progesterone. We are currently trying to troubleshoot these experiments and are hoping that in the future we will be able to repeat these experiments. In the meantime, we have used an in vivo approach to determine if PTHrP is involved in regulating hormonally stimulated growth at the TEB during puberty.

To do this, we have quantitated the rate of cellular proliferation and cell turnover in TEBs of 5 week-old normal and K14-PTHrP mice treated with and without hormones. The mice were injected intraperitonealy with both estrogen (24 ug/day) and progesterone (1.2 mg/day) for two days. These concentrations of hormones have been previously shown to cause ductal outgrowth in adolescent mice (Wysolmerski et al., 1996). Mice were then injected with 100 μCi tritiated- thymidine, and the tymidine was allowed to incorporate for 45 minutes. The mammary glands were then harvested , fixed in 4% paraformaldehyde, embedded in paraffin and sectioned a t 5 μm. The rate of cellular proliferation was determined by counting the number of cells per end bud that were in the S-phase of mitosis (>4 silver grains) over the total numbers of cells. For these experiments, 3 end buds per mouse were counted in 5 different mice for total of 15 end buds for each experimental condition. As shown in Figure 2A, wild type mice injected with hormones showed an increase in cellular proliferation (23%) as compared to untreated wild type mice (14%), demonstrating that the hormone injections did cause an increase in ductal proliferation in normal mice. In contrast, there was no significant increase in cellular proliferation in TEBs of K14-PTHrP mice treated with hormones (14%) versus untreated animals (13%). These data suggest that there a failure to respond to hormonally stimulated in TEB of K14-PTHrP mice.

We also measured the rate of cell turnover in the TEBs of 5 week-old WT and K14-PTHrP treated with and without hormones by quantitating the amount of apopotsis using TUNEL analysis. For these experiments, the rate of cell turnover was measured by counting the average number of cells per end bud that were positive for apoptosis over the total number of cells per end bud. A total of 3 end buds per mouse in 5 different mice were used for these experiments, totaling 15 end buds per experimental condition. As shown in Figure 2B, 3% of the cells per TEBs in wild type animals were positive for apoptosis versus only 1% in wild type animals that were treated with hormones. This suggests that perhaps in addition to increasing cellular proliferation, hormone treatment also reduced the rate of apoptosis a the TEB. However, this decrease in apoptosis at the TEB following hormone treatment did not occur in the K14-PTHrP mice. As shown in Fig 2B, 6% of the cells per end bud in untreated K14-PTHrP mice were dying by apoptosis, as compared to 9% in treated animals. In addition, there was a significant increase in the amount of apoptosis in the TEBs form K14-PTHrP mice than wild type mice. Together, these results demonstrate that the defect in ductal elongation during puberty in the K14-PTHrP mice is the results if a decrease in the rate of cellular proliferation and an increase in the rate of cell turnover at the TEBs. This suggests that PTHrP may be involved in controlling ductal outgrowth at the TEB by regulating hormonally stimulated growth at the TEB.

PTHrP has a dual role in the mammary gland

In addition to the defect in ductal elongation , the K14-PTHrP mice also show a defect in the branching pattern of the epithelial ducts during puberty. We have recently shown that the defect in ductal morphogenesis in the PTHrP-knockout mice is due to alterations in the differentiation of the dense mammary mesenchyme during embryogenesis. Based on these results, we were also interested in determining if the pubertal defects in the PTHrP-overexpressing mice were the result of early developmental changes in the mammary mesenchyme, or if they were due to continued exposure to PTHrP-overexpression at the TEB. To answer this question, we used the tetracycline off system originally described by Bujard and co-workers to control temporally and spatially PTHrP overexpression in the mammary gland. A schematic diagram illustrating this system is shown in Fig 3 . This system consists of two transgenes. The first makes the tet transactivator protein (tTA) under control of the human keratin -14 (K14) promoter. The second makes PTHrP under control of tet operator sequences. In the presence of tetracycline, a conformational change occurs in the tTa preventing its binding to tet operator sequence, and the target gene remains off. In the absence of tetracycline, tTA can bind to the tet operator sequences and the target gene is turned on.

To be sure that this system could accurately target transgene expression to the mammary gland, we first crossed the K14-tTA mice with mice in which the lacZ gene was used as a reporter gene in place of PTHrP. We found that the resultant double transgenic mice expressed B-galactosidase in the epithelial cells of the embryonic mammary bud as well as in the cap cells of the TEB during puberty (Fig. 4A). In addition, no B-galactosidase acivity was found in mammary glands of mice that were raised on tetracycline, demonstrating that the transgene is turned off in the presence of tetracycline.

In order to determine if this system could also target PTHrP overexpression in the mammary, we tested for the expression of the pTet-PTHrP transgene in K14-tetTa/pTet-PTHrP double transgenics by assaying for human growth hormone sequences in total cellular RNA prepared from whole mammary glands. The pTet-PTHrP transgene contains a human growth hormone sequence t its 3' end that is transcribed but not translated, and can therefore be used to assay for transgene expression at the RNA level. By RNase protection analysis, we found that human growth hormone sequences were detected in the mammary tissue of K14-tTA/pTet-PTHrP mice raised off of tetracycline, but not in mammary tissue of mice raised on tetracycline demonstrating that the transgene is expressed in double transgenics raised off of tetracycline (Fig. 4B). Further more, the levels of transgene expression in the K14-tetTa/pTet-PTHrP mice raised off of tetracycline was similar to the levels of expression in the original K14-PTHrP mice (data not shown).

In the original K14-PTHrP mice there were two aspects to the pubertal defect. First, there was a delay in the overall growth of the epithelial ducts through the mammary fat pad. Second, there were severe defects in the

branching pattern of the epithelial ducts, resulting in a much simpler sparser virgin gland. In order to determine of we could recapitulate the original K14-PTHrP phenotype using our tetracycline- regulated system, we analyzed mammary development in 7 week-old K14-tT/pTet-PTHrP female mice raised on tetracycline (never saw PTHrP- overexpression) and in double transgenic mice raised off of tetracycline (always saw PTHrP- overexpression). As shown by whole mount analysis in Fig. 5A, mammary glands from K14-tTA/pTet-PTHrP mice raised on tetracycline (never saw PTHrP-overexpression) appear completely normal. At this stage of development, the epithelial duct system had consistently reached the borders of the mammary fat pad, and had a highly organized complex branched architecture. In contrast, in double transgenic mice raised off of tetracycline (always saw PTHrP-overexpression), the overall growth of the epithelial ducts into the fat ad was delayed, and there was a significant reduction in the degree of side branching and many fewer tertiary and quaternary branching (Fig. 5B). Therefore, these results demonstrate that we were able to recapitulate both aspects of the phenotype of the original K14-PTHrP mice using the tetracycline –off system, and that these defects could be completely inhibited in the presence of tetracycline.

We next examined mammary development in K14-tTA/pTet-PTHrP mice that saw PTHrP overexpression only before birth (conception to birth), and in mice that saw PTHrP overexpression only after birth (birth to 7 weeks). As shown in Fig. 6A, at this stage of development, the duct system in normal mice has reached the borders of the mammary fat pad, and has undergone several rounds of branching morphogenesis resulting in a highly branched organization. In contrast , K14-tTA/pTet-PTHrP mice that saw PTHrP overexpression only after birth showed an impairment in the overall growth of the epithelial ducts through the mammary fat pad (Fig. 6B, Table 1), as demonstrated by the significant reduction in the length of the epithelial ducts (1.48 cm in WT; 0.92 cm in double transgenics) as well as a reduction in the percentage of the fat pad filled by the epithelial ducts (90% in WT; 59% in double transgenics). Interestingly, these mice exhibited only mild defects in the branching pattern of the epithelial ducts, as determined by measuring the average number of branch points per 5 mm^2 area (66 in WT; 49 in double transgenics) as well as the number of primary branches off of the main duct (18.5 in WT;17.5 in double transgenics) . In contrast, K14-tTA-pTet-PTHrP mice that saw PTHrP overexpression only before birth showed only mild defects in the growth of the epithelial ducts through the fat pad (length of ducts 1.48 cm in WT, 0.92 cm in double transgenics; percent of fat pad filled 90% in WT, 76% in double transgenics). However, these mice had severe defects in the branching growth of the epithelial ducts during puberty(Fig.6C, Table 1). These defects in branching growth appear to be due to differences in the degree of side branching, as shown by the reduction in the degree of overall branching (average number of branches in 5 mm^2 area: 66 in wild type, 29 in double transgencis) rather than defects in primary branching (average number of primary branches: 18.5 in WT, 13 in double transgenics).

Together, these results suggest that the change in the branching pattern in the K14-PTHrP mice is due to PTHrP-overexpression before birth, whereas the change in the rate of growth of the epithelial ducts through the mammary fat pad is related to PTHrP-overexpression after birth.

In addition to the pubertal defects in the original K14-PTHrP mice there were also defects in lobuloalveolar development during early pregnancy, characterized by the delayed formation of a reduced number of alveoli. However, it was unclear if these defects were simply the result of a reduced ductal mass due to the pubertal defects, or whether overexpression of PTHrP also impaired lobuloalveolar development . To distinguish between these possibilities, we once again turned to our tetracycline regulated system . For these experiments, K14-tetTA/pTet-PTHrP mice were raised on tetracycline (PTHrP-overexpression is off) until pubertal growth was complete (at about 8-10 weeks of age) and PTHrP-overexpression was then turned on by removing tetracycline form the drinking water. After two weeks, we allowed the mice to become pregnant, and examined lobuloalveolar development at day 15 of pregnancy. As shown in Fig. 7, the mammary glands from both the normal littermate and from the K14-tetTa/pTet-PTHrP demonstrated robust growth and the normal development of lobuloalveolar structures. This result suggests that the defects in lobuloalveolar development in the original K14-PTHrP mice are due to the reduced ductal mass resulting from changes in ductal growth during puberty and are not directly due to PTHrP overexpression during pregnancy.

Objective 3: Characterization of differentially expressed genes in embryonic mammary buds from normal and PTHrP null mice

The main goal of this objective was to define comprehensively the changes in gene expression in mammary stromal cells following PTHrP signaling. For these experiments, we performed subtractive hybridization on PTHrP-treated and untreated primary cultures of mouse mammary stromal cells. The basic method behind cDNA subtraction was as follows: Mammary stromal cells were treated with 10^{-7} M PTHrP for 6 and 24 hours. The time points 6 and 24 hours were chosen to detect genes that might be directly upregulated by PTHrP signaling (6 hours) and those whose upregulation may be secondary to PTHrP signaling (24 hours). The cells were then harvested and poly A+ mRNA was isolated using the Poly A+ Pure Kit from Ambion. cDNA subtraction was performed using the PCR-Select cDNA Subtraction Kit from Clontech. Briefly, the Poly A+ mRNAs from PTHrP-treated and PTHrP-untreated cells were converted into cDNA populations referred to as tester and driver, respectively. The two cDNA populations were then hybridized, and the hybridized sequences removed thereby eliminating cDNAs that are common to both populations of cells. The unhybridized cDNAs were then amplified by PCR and then blunt end cloned into a PCR-cloning vector and screened by dot blot analysis. For dot blot analysis inserts from the individual clones were amplified by PCR and blotted in groups of 96 onto nylon filters and hybridized to forward- and reverse-subtracted clones. The forward -subtracted probe was made from the same subtracted

cDNA library used to construct the library. To make the reverse subtracted probe, subtractive hybridization is performed with the original tester cDNA as driver and the driver cDNA as tester. Clones representing mRNAs that are truly differentially expressed will hybridize only with the forward-subtracted probe; clones that hybridize with the reverse-subtracted probe may be considered background.

As reported last year, we isolated 26 individual clones that represented genes that might be differentially regulated by PTHrP (Table 2). These clones were sequenced and the sequences were compared against the GenBank database to identify known genes. Fourteen of these clones represent previously identified genes that could be assigned to one of five categories: 1) extracellular matrix and associated molecules, 2) transcription/differentiation factors, 3) growth factors, 4) signal transduction proteins and 5) proteins that are thought to have intracellular functions. The remaining twelve clones represent unknowns.

To confirm that these clones represented truly differentially expressed genes, we performed RNase protection analysis on RNA from PTHrP-treated mammary stromal cells using the subtracted clones as probes. Unfortunately, only one out of the 26 clones turned out to be differentially expressed in response to PTHrP in mammary stromal cells. The other clones represented false positives. The one positive clone that we identified represents the gene for colony stimulating factor –1 (csf-1), a homodimeric serum growth factor that is involved in regulating mononuclear phagocytes. Mice carrying a mutation of the csf-1 gene (resulting in a non-functional truncated protein) have a mammary defect during pregnancy lactation, however embryonic development and adolescent development are normal . Because the csf-1 mutation does not phenocopy the PTHrP-knockout, it is unlikely that csf-1 is the primary downstream target of PTHrP in embryonic and adolescent mammary development.

During the course of these experiments, we had generated data demonstrating that , at least during embryogenesis, PTHrP is involved in the differentiation of the dense mammary mesenchyme. Therefore, it is possible that the adult mammary stromal cells, which are fully differentiated cells, would not give us insights into potential downstream signaling partners to PTHrP. We are planing on repeating these experiments using primary cultures of undifferentiated ventral mesenchymal cells from 12 day-old embryos. We have already begun to grow these cells in culture and have shown that they do express PPR1 and are able to respond to PTHrP with and increase in intracellular cAMP. Although these experiments are not within the scope of this proposal, we are hopeful that repeating these experiments with undifferentiated cells will allow us to identify downstream signaling partners to PTHrP.

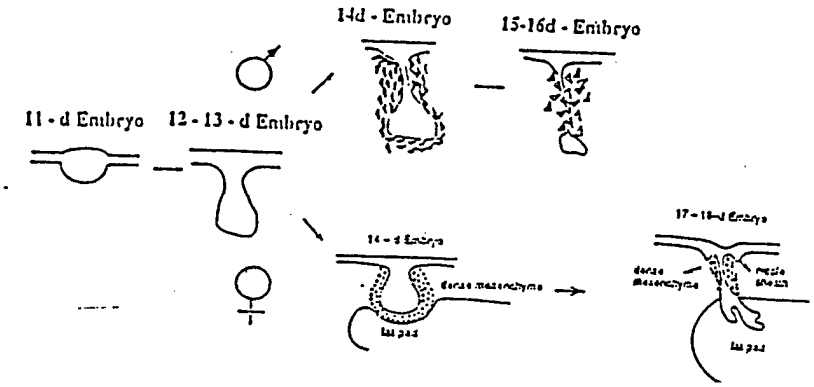

Figure 1. Outline of mammary development. Please see text for details.

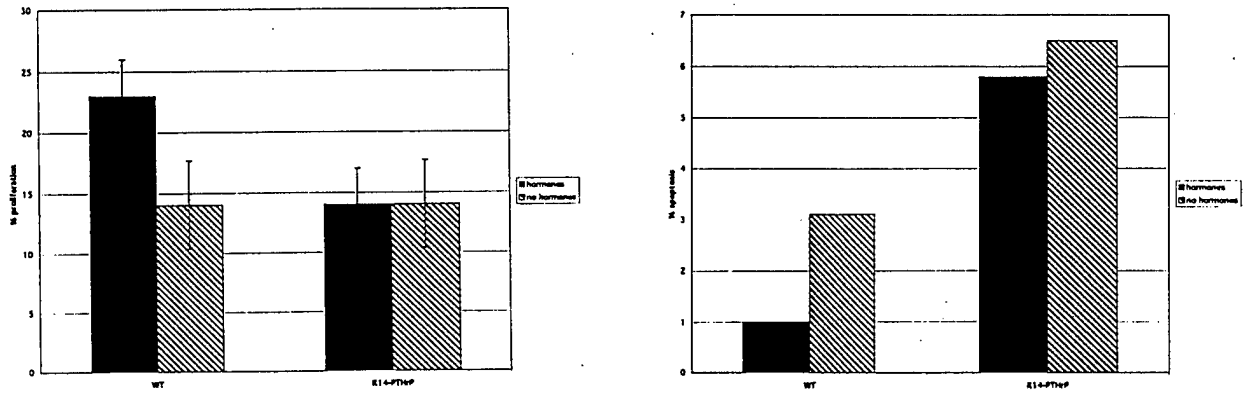

Figure 2. Quantitation of cellular proliferation and cell turnover in the end buds of WT and K14-PTHrP mice treated with and without hormones. A.) The rate of cellular proliferation in the end buds of hormone treated and untreated WT and K14-PTHrP. B.) Apoptosis in hormone treated and untreated WT and K14-PTHrP mice. For these experiments, 3 end buds per mouse from 5 different mice were used totaling 15 end buds per experimental condition.

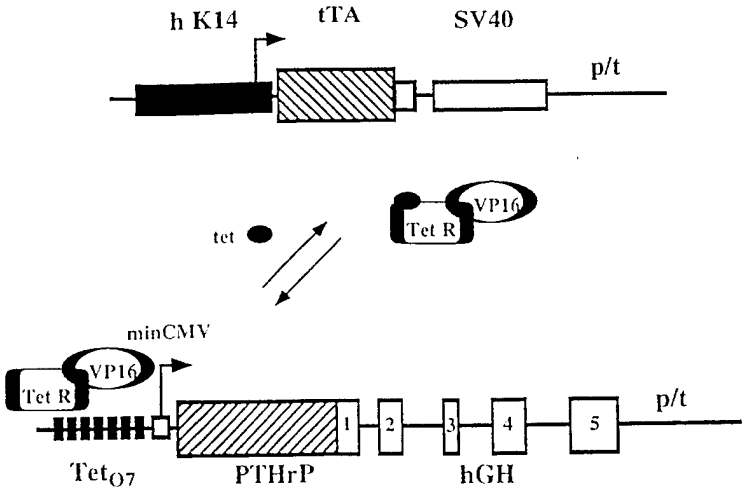

Figure 3. Schematic diagram of" tet-off"system. Please see text for details.

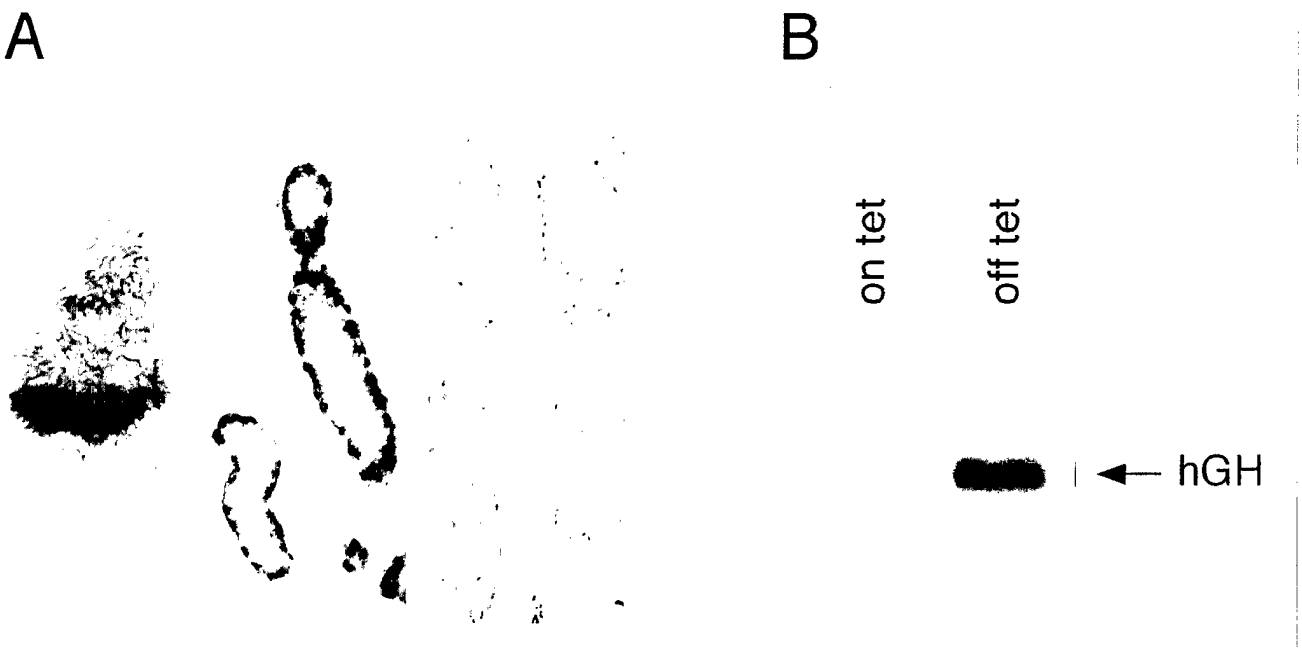

Figure 4. A.)Histological examination of ß-galactosidase activity in embryonic mammary bud and adult virgin mammary gland of K14-tTA/pTet-lacZ double transgenic mice. The left hand panel represent a section through a mammary bud of a 15 day old embryo raised off of tetracycline, the middle panel represent a section through the mammary gland of an 8-week old adult virgin mouse raised off of tetracycline, and the right hand panel represent a section through a mammary gland of an 8 week old mouse raised on tetracycline. Note that the epithelial cells of the embryonic mammary bud, as well as the myoepithelial cells of the adult virgin mammary gland stain blue demonstrating the presence of ß-galactosidase activity in animals raised of f tetracycline. In contrast, there is no ß-galactosidase activity in the mammary glands of animals raised on tetracycline. B) RNase protection analysis of total RNA from K14-tTA/pTet-PTHrP double transgenic mice raised on and off tetracycline. Note that there is expression of the transgene in mice that were raised off of tetracycline but not in animals raised on tetracycline.

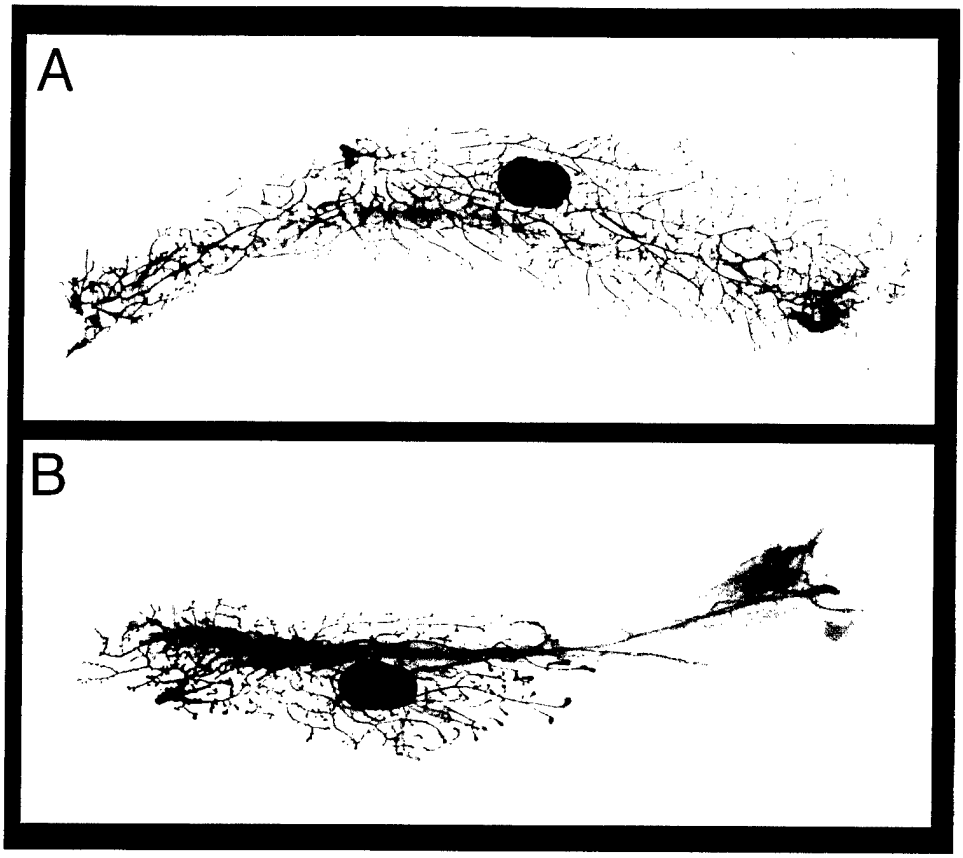

Figure 5. Whole mount analysis of mammary glands from K14-tTA/pTet-PTHrP mice that never s saw PTHrP overexpression (panel A) and K14-tTA/pTet-PTHrP mice that always saw PTHrP overexpression (panel B). Note that there are defects in both duct elongation and branching morphogenesis in the mammary glands of double transgenics that always saw PTHrP overexpression, while the mammary glands from double transgenics that never saw PTHrP overexpression

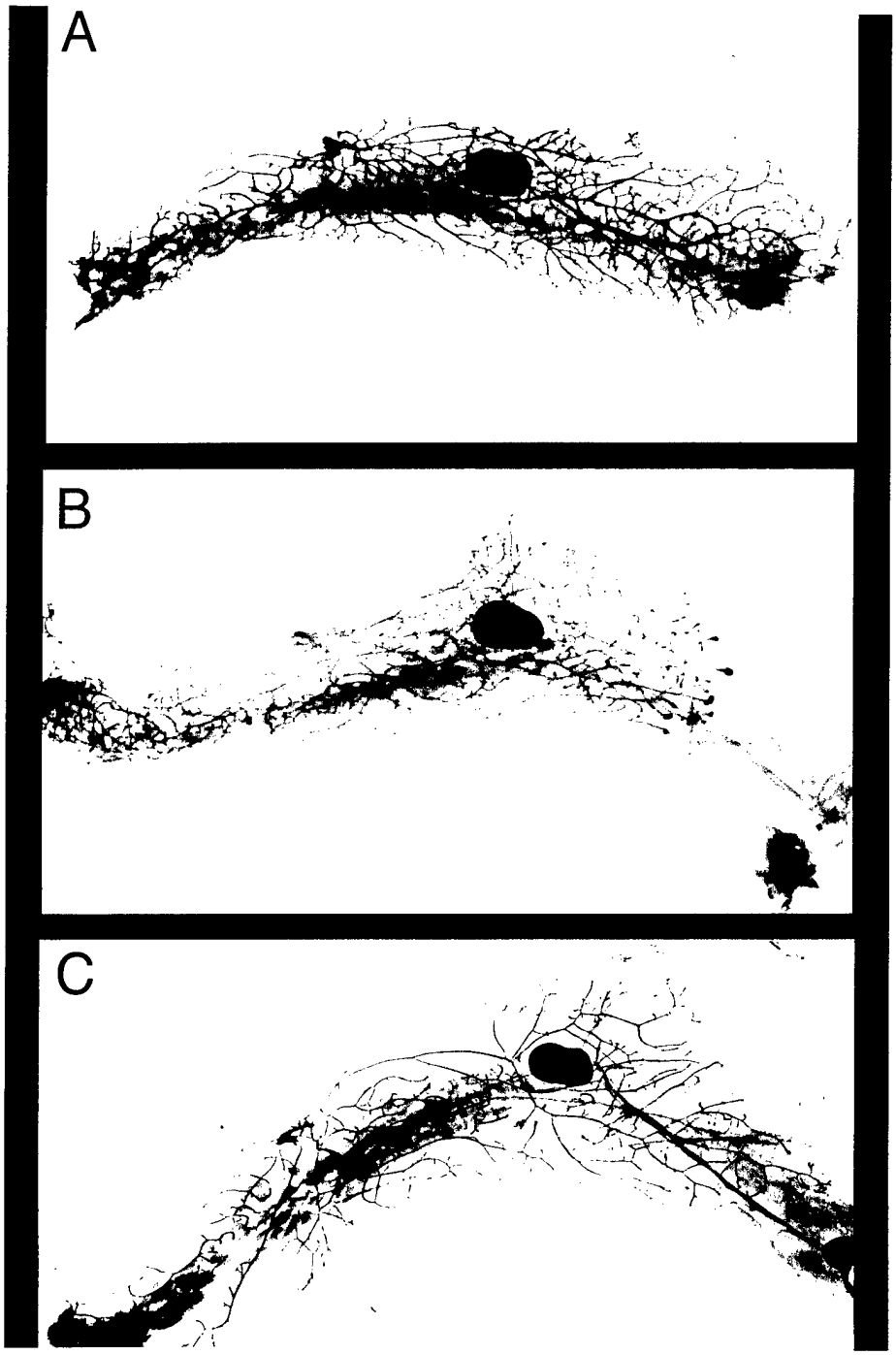

Figure 6. Whole mount analysis of mammary glands from normal mice (panel A) K14-tTa/pTet-PTHrP mice that saw PTHrP overexpression only after birth (panel B) and K14-tTA/pTet-PTHrP mice that saw PTHrP overexpression only before birth. Note that, as compared to the mammary gland from the normal mouse, double transgenics that saw PTHrP overexpression after birth had defects only in duct elongation during puberty, while mice that saw PTHrP overexpression before birth had defects in the branching pattern of the epithelial ducts during puberty.

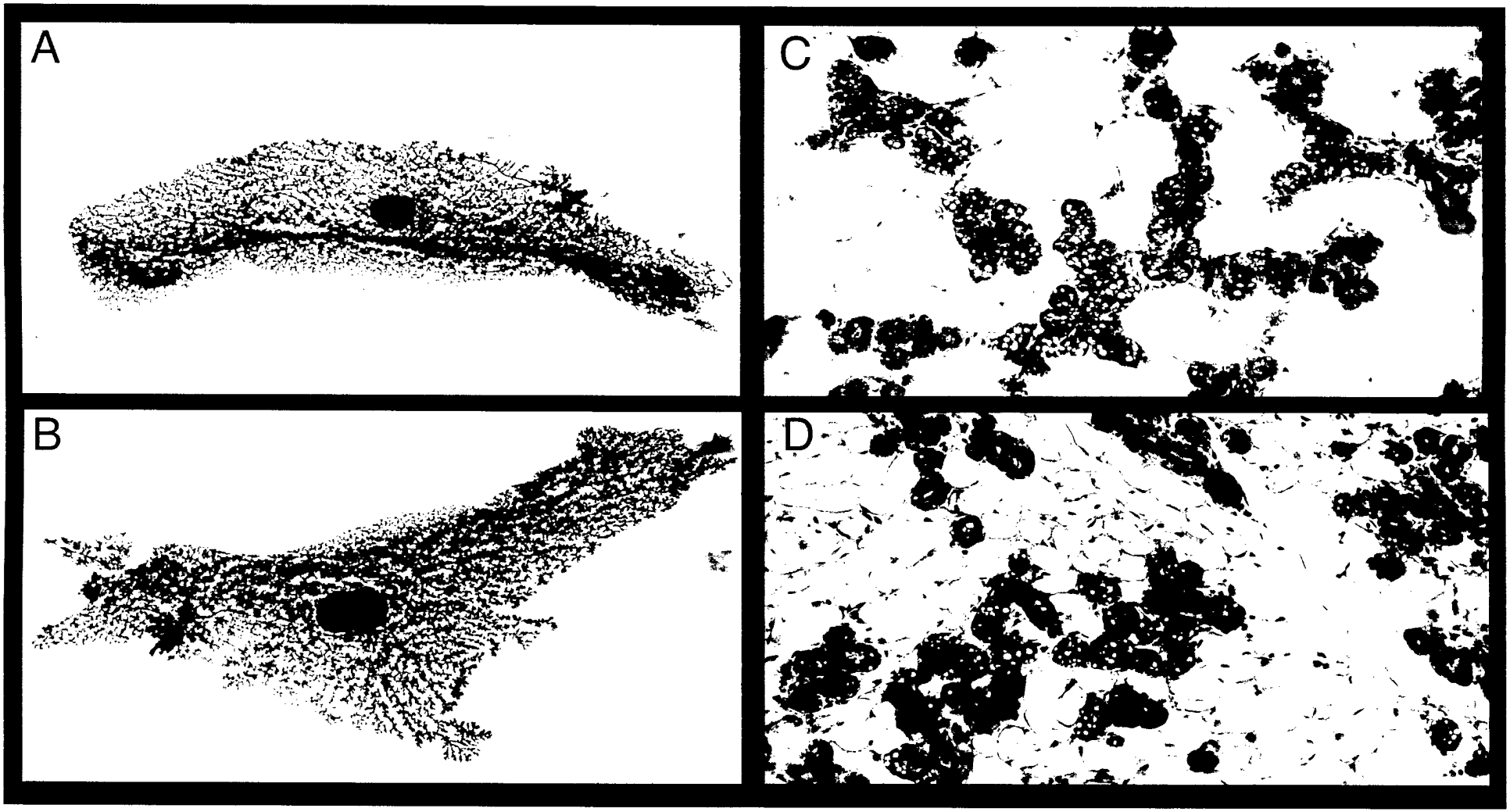

Figure 7. Whole mount analysis of mammary glands from 15 day pregnant normal (panels A and C) and K14-tTA/pTet-PTHrP mice (panels B and D). PTHrP overexpression remained off until 8 weeks of age to allow for normal pubertal development in these mice. PTHrP overexpression was then turned on for two weeks and the mice were allowed to become pregnant. Mammary development was examined at 15 days of pregnancy. Note that there are no differences in lobuloalveolar development in normal and double transgenic mice.

	Total Duct Length	% of FP penetration	Av # of branches per 5mm^2	Av # of branches off primary
WT	1.48 cm	90%	66	18.5
Dbl Tg after	0.92 cm	59%	49	17.5
Dbl Tg before	1.3 cm	76%	29	13

Table 1 – Histomorphometry of double transgenic mammary glands. Measurements were performed on 5 glands for each condition. Node analysis performed on 15, 5 mm2 area

Table 2- List of Clones Identified by Subtractive Hybridization of PTHrP-treated and untreated mamamry stromal cells

Cell Adhesion/Extracellular Matrix
stromelysin 1
PG-M Core Protein
ryudocan (syndecan 4)
type III collagen
fibrillin

Signal Transduction
14-3-3 zeta
ARF-related protein

Transcription factors
LZIP
msx-1 homologue

Growth Factors
colony stimulating factor-1
T1 protein (IL-6 receptor homologue)

Intracellular Calcium Associated Proteins
calpactin
calreticulin
ribophorin

Key Research Accomplishments

Over the three years of funding for this project we have shown that:

- PTHrP and its receptor, PPR1, are expressed in an epithelial-mesenchymal (stromal) pattern throughout mammary development.

- PTHrP and PPR1 are most predominately expressed in the embryonic mammary bud and in the terminal end bud during puberty, both sites of active cellular proliferation and differentiation in the mammary gland.

- PTHrP is involved in determining mesenchymal cell fate decision during embryogenesis and in its absence the ventral mesenchyme does not become mammary mesenchyme and mammary development fails.

- PTHrP signaling through the mammary stroma is necessary for epithelial ductal branching morphogenesis during embryogenesis

- PTHrP is involved in regulating hormonally stimulated growth at the TEB during puberty.

- PTHrP has a dual role in mammary development: Before birth, PTHrP is involved in regulating the subsequent pattern of epithelial branching. After birth, PTHrP is involved in regulating the rate of ductal elongation at the TEB during puberty.

Reportable Outcomes

Invited review articles

1. Dunbar ME and Wysolmerski JJ. (1999) Parathyroid hormone related protein: A developmental regulatory molecule necessary for mammary gland development. *Journal of Mammary Gland Biology and Neoplasia.* 4:21-34

2. Dunbar ME, Wysolmerski JJ, and Broadus AE. (1996). Parathyroid hormone related protein: from hypercalcemia of malignancy to developmental regulatory molecule. *American Journal of the Medical Sciences.* 312:287-294.

Peer-reviewed publications

1. Dunbar ME, Dann PR, Robinson GW, Hennighasuen L, Zhang J-P, and Wysolmerski JJ. (1999) Parathyroid hormone related protein signaling is necessary for sexual dimporhism during embryonic mammary development. *Development*, 126:3485-3493.

2. Dunbar ME, Young P, Zhang J-P, McCaughern-Carucci JF, Lanske B, Orloff J, Karaplis A, Cunha G, and Wysolmerski JJ. (1998) Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone related protein. *Developmental Biology* **203:**75-89.

3. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, and Broadus AE. (1998). Rescue of parathyroid hormone related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Development* **125:**1285-1294.

Abstracts

1. Dunbar ME, Dann PR, Dreyer BE, Broadus AE, Philbrick WM and Wysolmerski JJ. Transient early overexpression of PTHrP leads to subsequent defects in mammary development. Oral presentation: The Endocrine Society Annual Meeting, June 12-15. San Diego, California.

2. Dunbar ME, Dann PR, Robinson G, and Wysolmerski JJ. PTHrP Signaling is necessary for sexual dimporhism during fetal mammary development. Oral presentation: Second Joint Meeting of the American

Society for Bone and Mineral Research and International Bone and Mineral Society, December 1-6, 1998. San Francisco, California.

3. Dunbas ME, Young P, Zhang J-P, Orloff JJ, Karaplis A, Cunha G, and Wysolmerski JJ. Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by PTHrP. Poster presentation: Second Joint Meeting of the American Society for Bone and Mineral Research and International Bone and Mineral Society, December 1-6, 1998. San Francisco, California.

4. Dunbar ME, McCaughern-Carucci JF, Zhang J-P, Orloff JJ, and Wysolmerski JJ. The PTH/PTHrP receptor is expressed in mammary stromal cells throughout development. Poster presentation. The American Society for Cell Biology Annual Meeting, December 12-15, 1997. Washington DC.

Employment

The PI of this grant, Maureen Dunbar, Ph.D. has accepted the position of Assistant Professor at Penn State-Berks Lehigh Valley College in Reading Pennsylvania. This position begin August 15, 2000.

Personnel receiving pay from the research effort

Maureen E. Dunbar, Ph.D.

Conclusions

Over the course of this project, we have gathered evidence that supports our original hypothesis that PTHrP modulates mammary stromal cell function. First, we have shown that PTHrP and its receptor, PPR1 are expressed in an epithelial-mesenchymal pattern throughout the development of the mammary gland. Second, we have shown that during embryogenesis, PTHrP is involved in determining mesenchymal cell fate decisions, and in its absence the ventral mesenchyme can not become mammary mesenchyme. Third, we have shown that PTHrP signaling through the stroma is critical for ductal morphogenesis of the mammary epithelium during embryogenesis. These data demonstrate conclusively that it is PTHrP signaling through the mammary mesenchyme that is important for epithelial morphogenesis.

We have also provided evidence that PTHrP is involved in regulating hormonally stimulated growth at the TEB during puberty. We have shown that cellular proliferation is decreased and cell turnover is increased in K14-PTHrP mice treated with hormones. This suggests that PTHrP-overexpression may antagonize the effects of estrogen and progesterone stimulated growth during puberty. In addition, using a tetracycline regulated double transgenic mouse,

we have show that PTHrP-overexpression only before birth results in defects in the branching growth of the mammary epithelium during puberty, but not in defects in the rate of ductal elongation. Alternatively, PTHrP-overexpression after birth results in defects in ductal elongation but not in branching morphogenesis. These results are interesting because they suggest that PTHrP may have a dual role in the mammary gland. Before birth, PTHrP is involved in determining the subsequent branching morphogenesis of the mammary epithelium, while after birth, PTHrP is involved in regulating ductal elongation at the TEB.

Another goal of this project was to identify potential downstream signaling partners to PTHrP in the mammary gland. We approached this in two ways. First, we examined the expression of candidate growth factors in primary cultures of mouse mammary stromal c ells treated with PTHrP, and second, we performed subtractive hybridization on mammary stromal cells treated with PTHrP. Unfortunately, we were not able to identify any downstream signaling partners to PTHrP using these either of these techniques. As mentioned earlier, we now have reason to believe that the adult mammary stromal cells that we were using for these experiments were not the best source of tissue for these experiments as they are fully differentiated cells. Future experiments are planned in the laboratory using undifferentiated mesenchymal cells as a source of tissue to identify downstream signaling partners to PTHrP.

Although many of the experiments discussed in this final report were not part of the original proposal, we believe that the results generated from these experiments have given us a better understanding into the role of PTHrP in mammary gland development. With this new information in hand, we are now in a better position to begin to elucidate the molecular mechanisms by which PTHrP functions to regulate epithelial-mesenchymal interactions in the mammary gland.

References

Dunbar, M.E., and J.J. Wysolmerski. 1999. Parathyroid hormone-related protein: a developmental regulatory molecule necessary for mammary gland development. *Journal of Mammary Gland Biology and Neoplasia.* 4:21-33.

Sakakura, T. 1987. Mammary embryogenesis. *In* The Mammary Gland: Devlopment, Regulation and Function. M.C. Neville and C.W. Daniel, editors. Plenum Press, New York. 37-66.

Wysolmerski, J.J., J.F. McCaughern-Carucci, A.G. Daifotis, A.E. Broadus, and W.M. Philbrick. 1996. Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development.* 121:3539-3547.

Wysolmerski, J.J., W.M. Philbrick, M.E. Dunbar, B. Lanske, H. Kronenberg, A.

Karaplis, and A.E. Broadus. 1998. Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Development.* 125:1285-1294.

Appendix 1 – Attached Re-prints

1. Dunbar ME, Dann PR, Robinson GW, Hennighausen L, Zhang J-P, and Wysolmerski JJ. (1999) Parathyroid hormone related protein signaling is necessary for sexual dimorphism during embryonic mammary development. *Development*, 126:3485-3493.

2. Dunbar ME, Young P, Zhang J-P, McCaughern-Carucci JF, Lanske B, Orloff J, Karaplis A, Cunha G, and Wysolmerski JJ. (1998) Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone related protein. *Developmental Biology* **203:**75-89.

3. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, and Broadus AE. (1998). Rescue of parathyroid hormone related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Development* **125:**1285-1294.

Development 125, 1285-1294 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV6330

Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development

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Accepted 21 January; published on WWW 26 February 1998

SUMMARY

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product that causes humoral hypercalcemia of malignancy. PTHrP is now known to be widely expressed in normal tissues and growing evidence suggests that it is an important developmental regulatory molecule. We had previously reported that overexpression of PTHrP in the mammary glands of transgenic mice impaired branching morphogenesis during sexual maturity and early pregnancy. We now demonstrate that PTHrP plays a critical role in the epithelial-mesenchymal communications that guide the initial round of branching morphogenesis that occurs during the embryonic development of the mammary gland. We have rescued the PTHrP-knockout mice from neonatal death by transgenic expression of PTHrP targeted to chondrocytes. These rescued mice are devoid of mammary epithelial ducts. We show that disruption of the PTHrP gene leads to a failure of the initial round of branching growth that is responsible for transforming the mammary bud into the rudimentary mammary duct system. In the absence of PTHrP, the mammary epithelial cells degenerate and disappear. The ability of PTHrP to support embryonic mammary development is a function of amino-terminal PTHrP, acting via the PTH/PTHrP receptor, for ablation of the PTH/PTHrP receptor gene recapitulates the phenotype of PTHrP gene ablation. We have localized PTHrP expression to the embryonic mammary epithelial cells and PTH/PTHrP receptor expression to the mammary mesenchyme using in situ hybridization histochemistry. Finally, we have rescued mammary gland development in PTHrP-null animals by transgenic expression of PTHrP in embryonic mammary epithelial cells. We conclude that PTHrP is a critical epithelial signal received by the mammary mesenchyme and involved in supporting the initiation of branching morphogenesis.

Key words: Epithelial-mesenchymal interaction, Branching morphogenesis, Ectodermal dysplasia, Genetic rescue, Keratin 14, Organogenesis, PTH/PTHrP receptor, Mammary mesenchyme

INTRODUCTION

Parathyroid hormone-related peptide (PTHrP) was initially isolated from tumors causing the paraneoplastic syndrome of humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). Its name reflects the fact that PTHrP and parathyroid hormone (PTH) are the products of genes that have diverged from a common ancestor (Broadus and Stewart, 1994). Unlike PTH, which is produced only by the parathyroid glands and circulates as a classic peptide hormone that regulates systemic calcium metabolism, PTHrP is produced by a wide variety of fetal and adult tissues, does not circulate and exerts its actions locally (Broadus and Stewart, 1994). PTH and PTHrP retain a high degree of homology in their amino-terminal portions, and PTH and amino-terminal species of PTHrP have retained the use of a common G-protein-coupled receptor, the PTH/PTHrP receptor (Jüppner et al., 1991). PTHrP has also been shown to undergo post-translational processing to generate several other peptides, at least one of which has been demonstrated to have biological activity subserved by an as yet unidentified receptor distinct from the PTH/PTHrP receptor (Wu et al., 1996; Kovacs et al., 1996).

PTHrP has been implicated in the regulation of a variety of biological processes such as cell growth and differentiation, the regulation of pancreatic islet cell function, the regulation of smooth muscle tone and the facilitation of placental calcium transport (Philbrick et al., 1996). Although the exact physiological functions of PTHrP remain unclear in mature organisms, a series of recent experiments in transgenic mice has demonstrated that PTHrP serves important roles during fetal development. Disruption of the PTHrP and PTH/PTHrP receptor genes and overexpression of PTHrP in chondrocytes have shown that PTHrP regulates chondrocyte differentiation during endochondral bone formation (Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996; Weir et al. 1996). In the absence of PTHrP or the PTH/PTHrP receptor, chondrocytes appear to differentiate and ossify prematurely, resulting in a chondrodystrophy that leads to the neonatal death of the knockout mice. Overexpression of PTHrP in chondrocytes leads to the opposite phenotype, a profound delay in the differentiation of chondrocytes resulting in the birth of mice with a cartilaginous skeleton. In addition to effects in chondrocytes, PTHrP has been implicated as playing a role in epithelial-mesenchymal interactions during hair follicle and mammary gland development. Overexpression of PTHrP in keratinocytes (Wysolmerski et al., 1994) results in either a delay or failure of hair follicle initiation, and its overexpression in mammary myoepithelial cells (Wysolmerski et al., 1995) has been shown to impair mammary ductal development.

Shortly after its discovery, PTHrP mRNA was found to be expressed in the lactating mammary gland and PTHrP was found in high concentrations in milk (Thiede and Rodan, 1988; Budayr et al., 1989). The role of PTHrP during lactation remains obscure, but it is now clear that PTHrP is expressed at various stages during mammary gland development, and overexpression of PTHrP in myoepithelial cells has been shown to retard ductular growth and to impair side branching during sexual maturation as well as to inhibit the formation of terminal ductules during early pregnancy (Wysolmerski et al., 1995). In addition, PTHrP introduced directly into the mammary fat pads of normal mice has been shown to impair estrogen- and progesterone-induced ductular proliferation (Wysolmerski et al., 1995). Because the skeletal phenotypes of PTHrP underexpression and overexpression were exact opposites of each other, we hypothesized that PTHrP gene ablation might also lead to defects in ductular growth and or branching. However, because mammary development occurs to a great extent after birth, and because the PTHrP-knockout mice die at birth, in order to test this hypothesis, we needed to devise a strategy to rescue these mice from their neonatal demise. In this report, we describe our strategy for rescuing the PTHrP-null mice, and we demonstrate that PTHrP is essential for mammary gland development.

MATERIALS AND METHODS

Mouse strains and identification of knockout embryos.

The disrupted PTHrP allele (Karaplis et al., 1994) was progressively outbred onto a CD-1 background and mice heterozygous for this allele were mated to produce PTHrP-null embryos. The date of the appearance of a vaginal plug was considered to be day 0 of embryonic life. Embryos were removed from the uterus and genotyped with respect to the presence or absence of neomycin gene sequences and the presence or absence of an intact PTHrP-coding region (exon IV) by PCR, utilizing the following primer sets: wild-type murine PTHrP gene – forward 5′-GCTACTGCATGACAAGGGCAAGTCC and reverse 5′-CATCACCCACAGGCTAGCGCCAACT (421 bp product), and bacterial neomycin gene – forward 5′-GGAGAGGCTATTCG-GCTATGAC and reverse 5′-CGCATTGCATCAGCCATGATGG (315 bp product). This allowed the identification of wild-type, heterozygous and homozygous PTHrP-null embryos. The disrupted PTH/PTHrP receptor allele was progressively bred onto a Black Swiss background, and homozygous-null embryos were produced and identified in like fashion (Lanske et al., 1996). The PTH/PTHrP receptor primer pair utilized for this purpose amplified a 270 bp portion of the PTH/PTHrP receptor gene and consisted of the following sequences: forward 5′GCAGAGATTAGGAAGTCTTGGA and reverse 5′AGCCGTCGT-CCTTGGGAACTGT.

Col II-PTHrP/PTHrP-null mice were produced in the following fashion. The col II-PTHrP and PTHrP-null alleles were first bred onto a CD-1 background for several generations to minimize any potential exacerbating effects of their original different genetic backgrounds. Then col II-PTHrP transgenic mice were crossed to PTHrP-null heterozygotes to generate offspring carrying both the transgene and a PTHrP-null allele. These were again crossed to PTHrP-null heterozygotes to generate col II-PTHrP hemizygous, PTHrP-null homozygous mice (col II-PTHrP/PTHrP-null mice). The PTHrP-null allele was identified as outlined above. The col II-PTHrP transgene was identified in like fashion using the following primers that identified a 510 bp section of the murine procollagen II promoter/human PTHrP cDNA junction segment: forward 5′-TCTT-AGCATTCTTGGAGAAC and reverse 5′-ATCAGATGGTGAAGG-AAG.

K14-PTHrP/PTHrP-null embryos were produced by mating K14-PTHrP transgenic hemizygotes (Wysolmerski et al., 1994) with mice heterozygous for the PTHrP-null mutation. Offspring of this cross that were both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null gene were then crossed to mice heterozygous for the PTHrP-null allele to produce mice homozygous for a disrupted PTHrP gene and hemizygous for the K14-PTHrP transgene. The K14 transgene was identified as previously described (Wysolmerski et al., 1995).

K14-PTHrP, col II-PTHrP/PTHrP-null (double rescue) mice were produced as follows. We first created K14-PTHrP hemizygous, PTHrP-null heterozygous and col II-PTHrP hemizygous, PTHrP-null heterozygous mice as described above. These mice were then crossed to one another to generate K14-PTHrP/col II-PTHrP double transgenic, PTHrP-null homozygotes (double rescue) mice. The various alleles were identified as outlined above.

Each of the various types of embryos was also sexed based on the presence or absence of a 240 bp band amplified from the SRY gene using the following primers: forward 5′-CGG-GATCCATGTCAAGCGCCCCATGAATGCATTTATG and reverse 5′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT (Geise et al., 1994).

Histology/immunohistochemistry

Embryos were harvested by caesarean section and fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was then removed, and the embryonic mammary glands were identified using transmitted light and photographed under low magnification. Subsequently, the mammary glands were dissected from the ventral skin and embedded in paraffin. Serial 5 μm sections were cut and stained with hematoxylin and eosin for microscopic examination. Immunohistochemistry was performed using standard techniques. The mouse casein antibody is a rabbit polyclonal antibody (kind gift of B. Vonderhaar, NIH, Bethesda MD) and was used at a dilution of 1:200. All primary incubations were performed for 12 hours at 4°C.

and primary antibody binding was detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3′ diaminobenzidine as a chromagen. Slides were counterstained using hematoxylin. Apoptosis was detected by terminal deoxytransferase labelling (TUNEL assay) employing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Mannheim, Germany).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed on 5 μm paraffin sections of embryonic mammary glands as follows. Probes corresponded to a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the PTH/PTHrP receptor gene, as previously described (Weir et al., 1996). Sense and antisense riboprobes were generated from linearized fragments using an in vitro transcription kit (Promega, Madison, WI) in the presence of ^{35}S-UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 μg/ml in PBS for 17 minutes at room temperature), and acetylated with 0.25% acetic anhydride in the presence of 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 minutes. Sections were then rinsed in 2× SSC and incubated for 30 minutes in 0.66% N-ethylmaleimide (Sigma Chemical Co., St Louis, Mo) in 2× SSC, rinsed again in 2× SSC, dehydrated in graded alcohol, treated with chloroform for 5 minutes, rehydrated and then air dried. The probes (1.5×10^7 cts/minute/ml) were then hybridized to the samples for 17 hours at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 250 μg/ml tRNA, 100 μg/ml salmon sperm DNA and 50 mM DTT. After hybridization, sections were rinsed in 1× SSC and washed twice in 2× SSC/50% formamide for 5 minutes at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 minutes. Following two rinses in 2× SSC, sections were again washed in 2× SSC/50% formamide at 52°C for 5 minutes, dehydrated through graded ethanol, air dried and dipped in a 1:1 mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development, sections were counterstained with hematoxylin and mounted for microscopic examination.

RESULTS

Col II-PTHrP rescued PTHrP-null mice lack mammary glands

Disruption of the PTHrP gene by homologous recombination resulted in defects in skeletal development including inappropriate ossification of the costal cartilage, resulting in a shield chest and respiratory failure (Karaplis et al., 1994). Most other tissues appear to have developed normally in these mice, but the neonatal death of these animals had precluded a full examination of the role of PTHrP in sites, such as the mammary gland, which develop after birth. Because overexpression of PTHrP via a procollagen II-PTHrP (col II-PTHrP) transgene produced a skeletal phenotype reciprocal to that seen in the PTHrP-knockout mice (Weir et al., 1996; Karaplis et al., 1994), we reasoned that delivery of PTHrP to chondrocytes, via this transgene, might rescue the skeletal phenotype of the PTHrP-knockout mice and allow these animals to survive beyond birth. Our goal was to produce a mouse that lacked PTHrP in all tissues except cartilage, where it would be supplied by the col II-PTHrP transgene. To this end, we bred the col II-PTHrP transgene onto a PTHrP-null background to produce col II-PTHrP/PTHrP-null mice. These mice survived to maturity but suffered from multiple abnormalities including defects in the integument and its appendages, and failures of tooth eruption and mammary epithelial development, a phenotype reminiscent of the collection of human syndromes known as ectodermal dysplasias (Freire-Maia and Pinheiro, 1994). In this report, we detail the effects of the loss of PTHrP on mammary development.

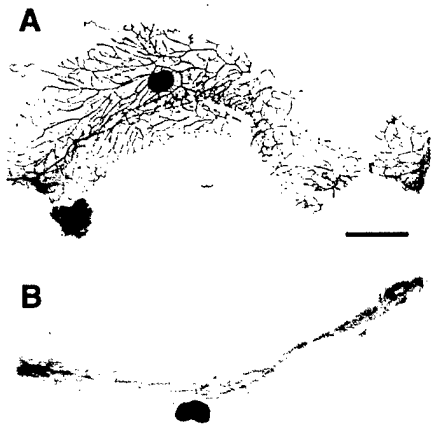

Fig. 1. Whole-mount analysis of mammary glands from col II-PTHrP/PTHrP-null mice and normal littermates. The fourth inguinal mammary glands were resected from 4-month-old normal and col II-PTHrP/PTHrP-null mice, fixed in acid ethanol and stained with carmine aluminum. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In contrast, the col II-PTHrP/PTHrP-null gland (B) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. These results are representative of the findings in 2 col II-PTHrP/PTHrP-null females. Scale bar represents 5 mm.

Fig. 1 demonstrates the morphology of whole mammary glands taken from 4-month-old, female col II-PTHrP/PTHrP-null (Fig. 1B) and normal littermate (Fig. 1A) mice. As one can see, the mature virgin mammary gland (Fig. 1A) consists of a series of branched epithelial ducts filling out a specialized stromal compartment known as the mammary fat pad. In contrast, col II-PTHrP/PTHrP-null mice lacked any evidence of mammary epithelial ducts (Fig. 1B). The mammary fat pad and its vasculature appeared to form normally, but were devoid of any mammary epithelium. Furthermore, examination of the ventral epidermis failed to demonstrate any nipple structures. These data suggested that PTHrP is essential for the development of the mammary epithelial duct system and nipples.

Loss of PTHrP results in a failure of the mammary epithelial primary growth spurt

The formation of the embryonic murine mammary gland is essentially a two-step process. The first step, occurring between E10 and E12, is the formation of the mammary buds. In female mice, the mammary buds remain relatively quiescent until E16 when they begin the second step, an initial round of branching morphogenesis, which leads to the formation of a mammary duct system with approximately 15-20 branches by

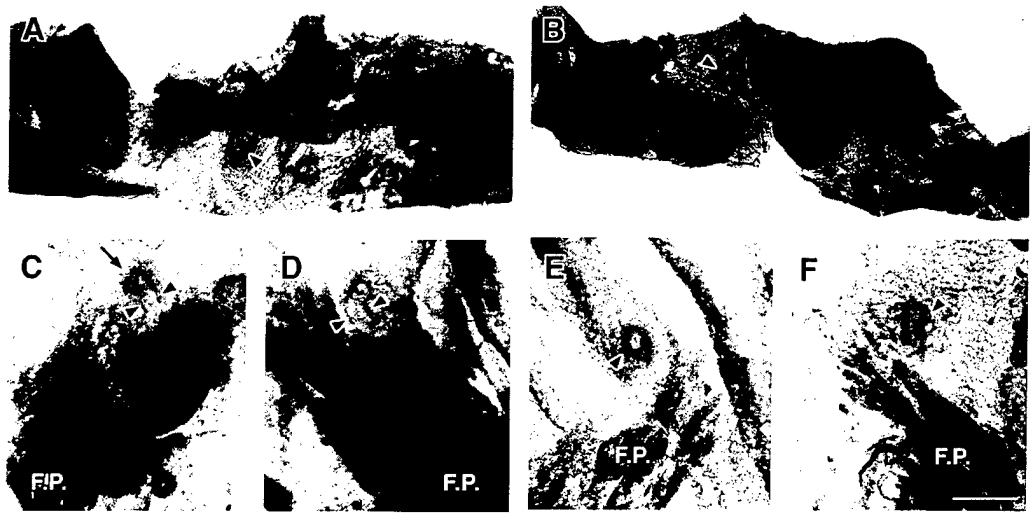

Fig. 2. Comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. The ventral epidermis was dissected from the respective embryos and photographed under low magnification using transmitted light in order to examine the gross structure of the embryonic mammary glands. (A,B) The mammary buds appear as round structures projecting upwards from the undersurface of the epidermis (arrowheads). Note that at E15, the mammary buds in the knockout embryos (B) appear similar to those in the normal embryos (A) (7 PTHrP-null female embryos analyzed, 6 wild-type embryos analyzed). In contrast, by E18 there is a dramatic difference in the appearance of the mammary structures in knockout (E,F) as compared to normal (C,D) embryos. At this point, the normal mammary structure consists of a developing nipple (dark halo, arrow in C) and an elongated primary duct (translucent tube-like structure between arrowheads in C,D) which is just beginning to form initial branches as it makes contact with the developing mammary fat pad (labelled F.P.). Note that in the PTHrP-knockout embryos (E,F), the mammary glands fail to elongate and remain bud-like (E) or slightly ectatic (F) in their appearance. There are no primary ducts that extend to the fat pads, and the developing fat pads (F.P.) themselves appear diminished in size (13 PTHrP-null female embryos examined, 6 wild-type embryos examined). Scale bar represents 160 μm for all panels.

birth (Sakakura, 1987). The nipples of mice are formed on or about E18 as a circular invagination of the epidermis, referred to as the nipple sheath (Sakakura, 1987). Because col II-PTHrP/PTHrP-null mice lack nipples and given the timing of nipple formation at E18, we reasoned that the loss of epithelial ducts resulting from the lack of PTHrP most likely occurred during the embryonic development of the mammary gland. Therefore, we returned to the original PTHrP-knockout embryos and examined embryonic mammary gland growth at days E12-13, E15 and E18, and at birth.

Figs 2 and 3 demonstrate the gross and microscopic appearance, respectively, of the mammary rudiments from mice homozygous for a disrupted PTHrP gene as compared to their wild-type littermates. As shown in Figs 2A,B and 3A,B, the mammary buds appeared normal in PTHrP-null embryos at E15. This was also the case at E12-13 (data not shown). In contrast, there was a dramatic difference in the appearance of the PTHrP-knockout ducts as compared to those in wild-type embryos at E18. As seen in Fig. 2C,D, at this age in the normal embryos, the mammary bud has given rise to a primary duct, which has elongated to make contact with the developing mammary fat pad and has formed several initial branches. In the knockout embryos, the mammary buds failed to make this transition and appeared similar to those at E15 (Fig. 2E,F). Furthermore, the mammary fat pads, although present, appeared somewhat diminished in size in the knockout embryos.

On microscopic examination, one could see that, by E18, the normal ducts had extended into the lower dermis and had formed initial branches that could be seen amongst the preadipocytes constituting the developing mammary fat pad (see Fig. 3C,D). At this point, the normal mammary glands also had well-developed nipple sheaths surrounding the origins of the primary ducts (Fig. 3C). In contrast, as seen in Fig. 3E, at E18 the PTHrP-knockout ducts appeared not to have undergone the primary growth spurt. Instead of extending to the fat pad and branching, epithelial ducts were uniformly found only in the upper portions of the dermis, where, typically, they were enveloped by a dense condensation of fibroconnective tissue. In addition, there was no evidence of nipple sheath development surrounding the origins of the epithelial ducts in the PTHrP-knockout embryos. When examined at higher magnification, the epithelial cells within the knockout ducts often appeared to be degenerating. As compared to normal epithelial cells (Fig. 3F), there was separation of the PTHrP-knockout epithelial cells (Fig. 3G) from the basement membrane, the cells borders were indistinct, and many nuclei appeared pyknotic. Consistent with this observation, by birth, there were only scattered remnants of degenerating mammary ducts that could be found on serial sectioning of the PTHrP-null embryos while, in wild-type embryos, the mammary ducts were firmly established within the mammary fat pad and had developed the expected branching pattern (data not shown). In summary, in the absence of PTHrP, mammary development proceeds normally through the mammary bud stage but subsequently falters as the buds fail to undergo the initial phase of branching morphogenesis and the mammary epithelial cells then degenerate.

PTHrP overexpression has been shown to delay chondrocyte differentiation and apoptosis, whereas disruption of the PTHrP

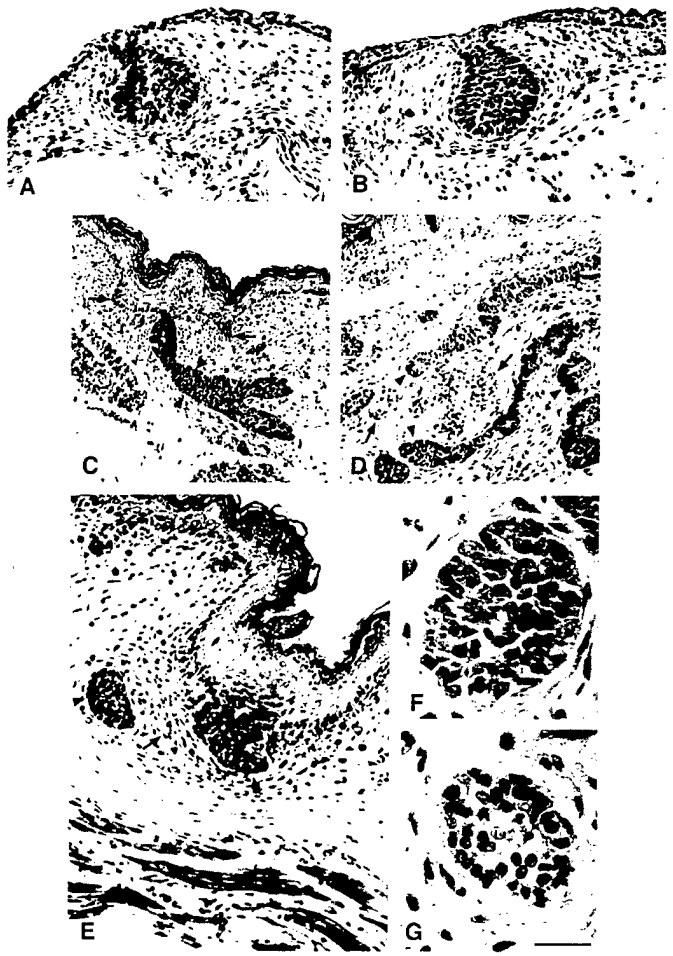

Fig. 3. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. (A,B) Photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15 (3 knockout and 3 wild-type embryos examined). At this stage, the mammary bud consists of an invagination of mammary epithelial cells surrounded by a condensation of mammary mesenchyme, and the microscopic appearance of the PTHrP-knockout buds was entirely normal. (C-G) Photomicrographs of H&E-stained sections through mammary glands dissected from PTHrP-knockout (E,G) and normal littermate (C,D,F) embryos at E18 (5 knockout and 4 wild-type embryos examined). In a normal embryo (C,D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad). In contrast, in the PTHrP-knockout embryos, (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). (F,G) High power photomicrographs of mammary epithelial ducts in cross-section taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that, in the knockout duct (G), the epithelial cells appear to be degenerating; many nuclei are pyknotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are separating from the basement membrane. Scale bar represents 16 μm in A,B; 25 μm in C,D; 17 μm in E; 5 μm in F,G.

gene results in a form of growth failure associated with premature differentiation and apoptosis of chondrocytes in the growth plate of developing bones (Weir et al., 1996; Amling et al., 1997; Karaplis et al., 1994; Lee et al., 1996; Vortkamp et al., 1996). Given these findings and the apparent degeneration of the mammary epithelial cells in the PTHrP-knockout embryos, we examined these cells for evidence of apoptosis and/or inappropriate differentiation at E18 by TUNEL assay and by immunohistochemistry for β-casein. Although apoptotic cells were easily detected in normal ducts, there were no apoptotic cells within the knockout ducts (data not shown). Likewise, immunohistochemistry for β-casein revealed no evidence that the knockout mammary epithelial cells were undergoing premature cytodifferentiation; there was no staining for β-casein in either normal or knockout epithelial cells at E18 (data not shown). These data suggest that, unlike the events in cartilage, the failure of mammary development in PTHrP-knockout mice appeared neither to be associated with premature differentiation of the mammary epithelial cells nor with widespread apoptosis of these cells.

Ablation of the PTH/PTHrP receptor gene recapitulates the mammary phenotype of PTHrP-knockout mice

As mentioned in the Introduction, PTHrP gives rise to several biologically active peptides (Broadus and Stewart, 1994; Wu et al., 1996). In addition, it has been suggested that PTHrP may be targeted to the nucleus and exert biological activity via an intracrine pathway (Henderson et al., 1995). Previous experiments had implicated soluble, amino-terminal PTHrP acting via the PTH/PTHrP receptor as important in the regulation of branching morphogenesis in the mammary gland during sexual maturation and pregnancy (Wysolmerski et al., 1995). In order to determine if this was also the case during embryogenesis, we examined mammary gland development in PTH/PTHrP receptor-null embryos (Lanske et al., 1996) over the same time frame as in the PTHrP-null embryos.

Fig. 4 demonstrates the appearance of the mammary rudiment in PTH/PTHrP receptor knockout mice and control littermates. As seen in the PTHrP-knockout embryos, in the receptor-knockout mice, the primary round of branching morphogenesis failed, leading to the subsequent degeneration of the mammary epithelial ducts. Just as with the PTHrP knockouts, the mammary buds appeared to form appropriately in the receptor knockout mice (data not shown), but clear differences in the appearance of the receptor-knockout mammary rudiment as compared to normal littermates were apparent by E18. As shown in Fig. 4, by E18, the normal duct system (Fig. 4A,D) had grown to the fat pad and begun to branch, while the knockout mammary duct failed to elongate or branch and remained bud-like in its appearance (Fig. 4B). Examination at higher magnifications revealed that the mammary ducts in the receptor knockout mice (Fig. 4C,E) remained in the upper dermis, were enveloped within an abnormal condensation of stroma and appeared to be degenerating, a picture nearly identical to that seen with mammary ducts devoid of PTHrP (see Fig. 3). Furthermore, as with the absence of PTHrP, the receptor knockout embryos formed no nipple sheath (see Fig. 4B). Therefore, ablation of PTHrP or the PTH/PTHrP receptor led to the same phenotype,

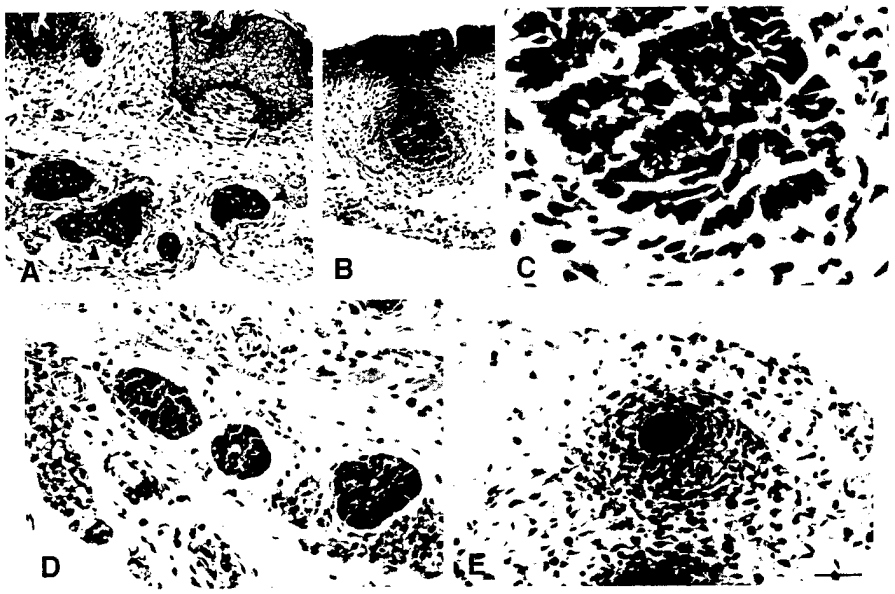

Fig. 4. Histologic comparison of the embryonic mammary glands of PTH/PTHrP receptor-knockout and normal littermate embryos at E18. (A) Photomicrograph of H&E-stained sections of mammary rudiment from a normal littermate. Note the initial branches of the primary duct (arrowheads) within the lower dermis. Also, note the developing nipple sheath (arrow) (3 embryos examined). (B) Photomicrograph of H&E-stained sections of mammary rudiment from PTH/PTHrP receptor-knockout embryo (5 receptor-knockout embryos examined). Note that the mammary duct has not elongated, that the mammary rudiment remains bud-like in its appearance and that there is no nipple sheath. (C) Higher magnification of B. Note that the epithelial cells appear to be degenerating; there are many pyknotic nuclei and the cell borders are indistinct, similar to the appearance of the PTHrP-knockout epithelial cells at this time point. (D,E) H&E-stained cross-sections of epithelial ducts from normal (D) and PTH/PTHrP receptor-knockout (E) mammary glands at E18. Note the lacy, delicate appearance of the stroma surrounding the normal ducts (D) as they make contact with the mammary fat pad. In contrast, note the condensation of stroma surrounding a rare PTH/PTHrP receptor-knockout duct (E) that has attempted to grow out from the mammary bud. Scale bar represents 20 μm in A,B; 4.5 μm in C; 10.4 μm in D,E.

a failure of the initial phase of branching morphogenesis during embryonic mammary development.

Localization of PTHrP and PTH/PTHrP receptor gene expression during embryonic mammary gland development

We next determined the sites of PTHrP and PTH/PTHrP gene expression in normal Balb/c mammary rudiments from E12 through E18 by in situ hybridization. As shown in Fig. 5A-C, PTHrP mRNA expression in the developing mammary rudiment was limited to the epithelial cells, especially those cells located peripherally, adjacent to the basement membrane. PTHrP mRNA was also detected in keratinocytes within the epidermis as well as within developing hair follicles, although it appeared that the highest levels of expression were within the mammary epithelial structures. Expression of the PTHrP

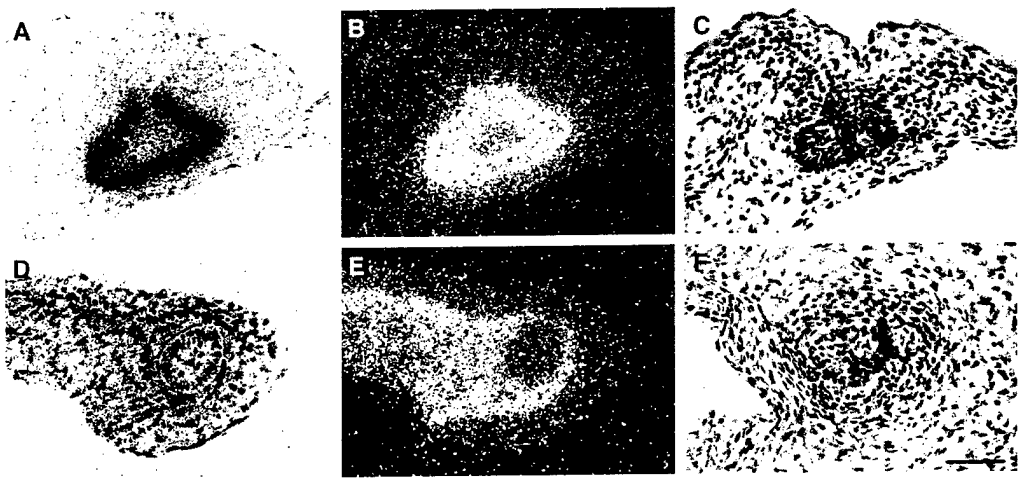

Fig. 5. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. (A-C) In situ hybridization for PTHrP mRNA in normal mammary rudiments at E16. (A,B) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. (C) Bright-field image of a similar section hybridized to sense probe as a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare A and C). (D-F) In situ hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. (D,E) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. (F) Bright-field image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to D. Scale bar represents 15 μm for all panels.

gene did not appear to be induced at any specific point during the time period that we examined (E12-18). Rather, PTHrP mRNA was continuously expressed at high levels in mammary epithelial cells in the mammary bud as well as in the growing ducts during the initial phase of branching morphogenesis.

In contrast to the epithelial expression pattern seen for PTHrP, expression of the PTH/PTHrP receptor was limited to the mesenchyme. As seen in Fig. 5D-F, PTH/PTHrP receptor mRNA was expressed throughout the embryonic dermis, including the dense mammary mesenchyme. At E12-13, the expression of the receptor mRNA appeared to be fairly uniform throughout the dermal mesenchyme (data not shown), but, from E15 onward, there appeared to be more intense hybridization of the receptor antisense probe in the upper, more cellular dermis (Fig. 5E). At E18, at a point at which the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in the stromal cells surrounding the growing mammary ducts as they became surrounded by the developing fatty stroma (data not shown). As with PTHrP gene expression, the PTH/PTHrP receptor gene was expressed throughout the time frame examined, and there was not a specific point at which its expression appeared to be induced. Therefore, within the embryonic mammary gland, PTHrP and the PTH/PTHrP receptor appear to represent an epithelial/mesenchymal signalling unit in which PTHrP is produced by mammary epithelial cells and interacts with its receptor on mammary mesenchymal cells.

Transgenic expression of PTHrP rescues the mammary glands of PTHrP-knockout mice

We hypothesized that the failure of mammary development seen in the PTHrP and PTH/PTHrP receptor-knockout embryos was due to the loss of PTHrP-mediated paracrine signalling between the mammary epithelium and mammary mesenchyme. This working hypothesis suggested that reintroducing PTHrP into the local microenvironment of the mammary bud might prevent the failure of mammary development in these mice. Keratin-14 expression is known to be induced in embryonic skin beginning at E15-16 (Kopan and Fuchs, 1989), about the time of the primary growth spurt of the mammary rudiment. Furthermore, the keratin-14 gene had been shown to be expressed in epithelial cells in the adult mammary gland (Smith et al., 1990; Wysolmerski et al., 1995). Therefore, we examined K14 expression in the embryonic mammary gland and found that it was expressed uniformly in embryonic mammary epithelial cells beginning on or about E15 (data not shown). Since we had shown that the K14-PTHrP transgene faithfully reproduced the native pattern of K14 expression in the mature mammary gland (Wysolmerski et al., 1995), we used this transgene as a vehicle to reintroduce PTHrP into the mammary environment of the PTHrP-null mice. We took a two-tiered approach. First, in order to ascertain if replacement of PTHrP into mammary epithelial cells rescued embryonic mammary development, we bred the K14-PTHrP transgene onto a homozygous PTHrP-null background to produce K14-PTHrP/PTHrP-null mice. These mice were devoid of PTHrP in all tissues except for those expressing the K14 gene. Second, in order to examine the effects of PTHrP replacement on the subsequent development of the mammary duct system within adolescent mice, we bred

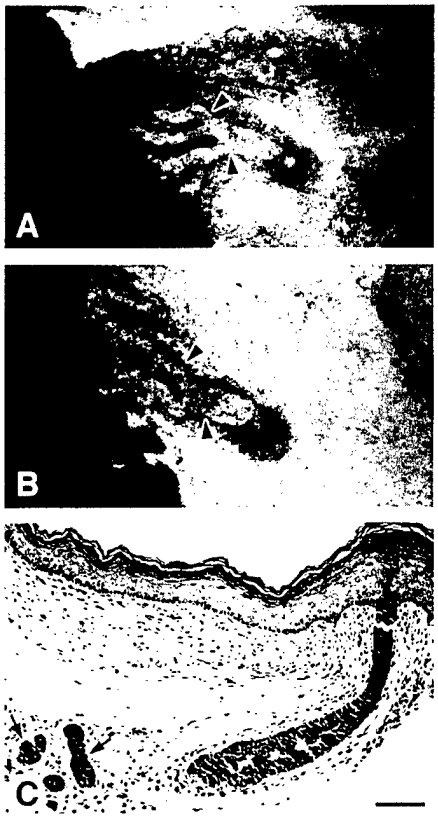

Fig. 6. Mammary development in K14-PTHrP/PTHrP-null mice. (A,B) Photographs of the whole mammary glands of a normal (A) and K14-PTHrP/PTHrP-null neonate (representative of 2 K14-PTHrP/PTHrP-null animals analyzed). The ventral skin was dissected and the mammary glands were viewed under low magnification using transmitted light. In both cases, the mammary gland consists of a primary duct (between arrowheads) that forms several branches before entering the mammary fat pad (dark area to the left). (Contrast B with the lack of a primary duct in the original PTHrP-null embryos as shown in Fig. 1E,F) (C) Photomicrograph of an H&E-stained section through the mammary gland of a K14-PTHrP/PTHrP-null neonate. Note that the primary duct extends from the epidermis through the dermis and forms its initial branches (arrows), as occurs in normal mammary development. However, despite the rescue of mammary epithelial development, note the lack of a nipple sheath. Scale bar represents 67 μm in A,B; 17 μm in C.

both the col II-PTHrP and K14-PTHrP transgenes onto a homozygous PTHrP-null background to produce col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. These double-rescue mice lacked PTHrP in all tissues except for chondrocytes and sites of K14 expression.

As expected, the K14-PTHrP/PTHrP-null mice died at birth due to the skeletal abnormalities resulting from the lack of chondrocyte PTHrP expression but, as opposed to the original PTHrP-knockout mice, these mice had mammary glands. As described in the previous sections, by birth, the epithelial duct system in the PTHrP-knockout embryos had completely degenerated. In contrast, as seen in Fig. 6, at birth, the K14-PTHrP/PTHrP-null mice had a well-formed primary duct that extended into the mammary fat pad and formed the expected initial branches. Interestingly, grossly, the primary ducts in the

K14-PTHrP/PTHrP-null neonates often appeared somewhat dilated as compared to normals. On H&E section, one could see that the epithelial duct system in the K14-rescued mice had extended below the upper dermis and, although the primary ducts again often appeared somewhat dilated histologically, they formed normal-appearing secondary ducts within the fatty stroma of the mammary fat pad (see Fig. 6C). Of note, despite the near normal appearance of the ductal tree, there remained no nipple sheath, as was also the case in the PTHrP-null embryos (compare Figs 3E and 6C). Therefore, expression of PTHrP in the embryonic mammary cells of PTHrP-null embryos under the control of the K14 promoter allowed the mammary bud to undergo the primary growth spurt but did not rescue nipple sheath formation.

The double-rescue mice lived to maturity in similar fashion to the col II-PTHrP/PTHrP-null mice. Although the double-rescue mice also lacked nipples, they had a mammary duct system. Fig. 7A demonstrates the fourth and fifth inguinal mammary glands taken from a mature double-rescue female. As can be seen, in these mice the reintroduction of PTHrP via the K14 transgene resulted in the successful completion of the initial round of branching morphogenesis and the appropriate extension of the mammary duct system into the mammary fat pad (Fig. 7A,B). However, the resultant duct system appeared to be that of a sexually immature animal. We had observed that female col II-PTHrP/PTHrP-null mice suffer from a form of hypothalamic hypogonadism (unpublished observations), and we hypothesized that this might have impaired the development of the mammary glands in the double-rescue mice. To address this issue, adult, double-rescue females were treated with subcutaneous estrogen and progesterone for 2 weeks. As shown in Fig. 7C,D, ductal growth in the mammary glands of hormonally treated double-rescue females progressed to the borders of the mammary fat pad and was appropriately branched. Therefore, replacement of PTHrP expression in the developing mammary gland via the K14 promoter was sufficient to support the early morphogenesis of the ductal epithelium and to allow for its subsequent growth and ramification.

DISCUSSION

This report records a series of observations that clearly demonstrate that amino-terminal PTHrP is required for the development of the mammary epithelial duct system in mice. First, col II-PTHrP/PTHrP-null mice (devoid of PTHrP in all tissues except for cartilage) lack all mammary epithelial ducts. Second, in PTHrP-knockout embryos, we found a primary failure of branching morphogenesis during embryonic mammary gland development. Third, deletion of the PTH/PTHrP receptor recapitulated the failure of mammary development seen in the PTHrP-knockout embryos. Finally, reintroduction of PTHrP into mammary epithelial cells via the K14-PTHrP transgene rescued the failure of embryonic mammary development seen in the absence of PTHrP and allowed the subsequent development of the mature mammary duct system within the mammary fat pad.

The formation of the embryonic mammary gland occurs in two steps: first, the formation of the mammary bud and, second, the initiation of branching morphogenesis that leads to the formation of the immature ductal tree (Sakakura, 1987). In PTHrP-knockout embryos, the mammary buds formed appropriately, but they failed to undergo the transition successfully into the initial round of branching growth that leads to the typical immature ductal tree. In the absence of PTHrP, the mammary epithelial structures failed to elongate and/or penetrate into the developing fat pad, remaining in the upper dermis and becoming surrounded by a dense condensation of fibroconnective tissue. The mammary epithelial cells subsequently degenerated; the nipple sheath failed to form, and, by birth, all traces of the mammary epithelial duct system disappeared, explaining the lack of mammary structures in the mature col II-PTHrP/PTHrP-null mice. The exact nature of the epithelial cell degeneration in the PTHrP-knockout embryos remains unclear. PTHrP has been shown to regulate chondrocyte differentiation and apoptosis in the developing growth plate (Weir et al., 1996; Amling et al., 1997; Lee et al., 1996; Vortkamp et al., 1996). However, the loss of the mammary epithelial cells in PTHrP-null embryos did not appear to be associated with either their premature differentiation (as measured by β-casein expression) or apoptosis. Histologically, the stromal condensation around the degenerating ducts in the PTHrP-null mice is reminiscent of the androgen-mediated stromal reaction that leads to the deterioration of the mammary rudiment in male embryos (Sakakura, 1987; Kratochwil and Schwartz, 1976). Despite this similarity, in female knockout embryos, the mammary buds appeared normal through E15, a point at which the mammary buds in normal male littermates are actively degenerating. This asynchrony makes it unlikely that modulation of PTHrP secretion and/or PTH/PTHrP receptor signalling is a central feature of the response of the mammary bud to fetal androgens. However, it remains a possibility that alterations in PTHrP signalling might play some role in the deterioration of the mammary epithelial cells in normal male embryos and we are currently pursuing a series of experiments to test this possibility.

The formation of the embryonic mammary gland is a classic example of inductive development involving epithelial-mesenchymal interactions (Sakakura, 1991; Cunha, 1994). Both the formation of the mammary bud and the initial round of branching morphogenesis appear to be critically dependent on a series of reciprocal and sequential signals exchanged between the mammary epithelium and the dense mammary mesenchyme (Thesleff et al., 1995; Cunha, 1994; Cunha et al., 1995; van Genderen et al., 1994; Weil et al., 1995, Yang et al., 1995). Several experiments have suggested that the presumptive mammary epithelium plays an important role in promoting the condensation and formation of the dense mammary mesenchyme (van Genderen et al., 1994; Kratochwil et al., 1996, Thesleff et al., 1995). However, once formed, the mammary mesenchyme appears to direct the formation of the mammary epithelial duct structure as well as to contribute to mammary epithelial cell cytodifferentiation. For example, heterotypic recombination experiments have demonstrated that mesenchymal cells from the fetal mammary gland can induce nonmammary epithelial cells to form mammary ducts and to make milk proteins (Cunha et al., 1995) and can even induce the formation of mammary bud-like structures from the epidermis of non-mammalian species (Propper, 1973; Propper and Gomot, 1973). Likewise, recent studies have demonstrated

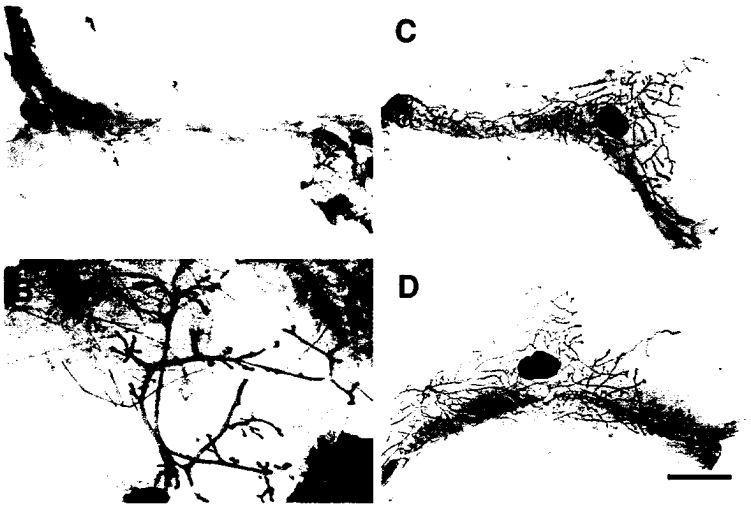

Fig. 7. Mammary development in col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. (A) Whole-mount analysis of the 4th and 5th inguinal mammary glands taken from a mature double-rescue female mouse (representative of 3 double-rescue mice examined). As one can see, epithelial ducts are present and have penetrated into the fat pad, but the duct appears sexually immature. (B) Higher magnification of 5th inguinal gland shown in A. (C,D) Whole-mount analysis of a mature normal gland (D) and an estrogen- and progesterone-treated double rescue gland (representative of 2 hormonally treated double-rescue mice). As can be seen after 2 weeks treatment of daily 17 β-estradiol (1mg/day) and progesterone (1 ng/day), the double rescue epithelial ducts (C) have grown to fill out the fat pad and appear similar to normals (D). Scale bar represents 5 mm in A and B, and 1 mm in C and D.

that signals derived from mesenchymal cells are important in regulating the overall rate of ductular proliferation as well as the pattern of branching that occurs during the process of branching morphogenesis (Yang et al., 1995; Witty et al., 1995; Phippard et al., 1996). We have demonstrated that, during embryonic mammary development, PTHrP gene expression is limited to the mammary epithelium while PTH/PTHrP receptor gene expression is restricted to the mesenchyme. In the context of the phenotype discussed above, these findings suggest that PTHrP acts as an epithelial message that must be received by the mammary mesenchyme in order for it to support branching growth.

Although mammary development does not appear to be abnormal in the PTHrP knockout embryos until E15-16, we have found that the PTHrP and the PTH/PTHrP receptor genes appear to be expressed in the mammary bud from its formation, at E12, onward. Furthermore, our K14 transgene crossing experiment suggests that PTHrP is largely dispensable before E15. K14 expression does not appear before this point, and therefore the mammary epithelium in the K14-PTHrP/PTHrP-null mice does not produce PTHrP before E15. Despite this delay in PTHrP secretion, as compared to normal mice, K14-PTHrP/PTHrP-null mice successfully initiate branching growth of the mammary ducts. This would imply that the critical period of PTHrP signalling for initiating branching morphogenesis is just before the primary growth spurt at E15-16. However, since nipple sheath development was not rescued in the K14-PTHrP/PTHrP-null mice and since the primary duct did not appear to be completely normal, PTHrP most likely also exerts earlier effects on the mesenchyme. Future study of the effects of PTHrP on mammary mesenchymal cells should help to clarify the details of the temporal requirements for PTHrP signalling during embryonic mammary development.

In summary, we have found that, during embryonic mammary gland development, PTHrP is a necessary participant in the epithelial-mesenchymal interactions leading to the formation of the rudimentary epithelial duct system. Specifically, PTHrP is produced by the mammary epithelium and appears to act on the mesenchyme, allowing it to support the initiation of branching morphogenesis. We have previously reported that the overexpression of PTHrP in mammary myoepithelial cells had dramatic effects on the process of branching morphogenesis during sexual maturation and pregnancy (Wysolmerski et al., 1995), indicating that PTHrP likely plays an important role in the regulation of this process throughout mammary development. There is also growing evidence of the participation of PTHrP in the reciprocal epithelial-mesenchymal interactions that govern epithelial development in sites other than the mammary gland. For example, the pattern of epithelial PTHrP expression and mesenchymal PTH/PTHrP receptor expression seen in the developing mammary gland has been noted in other developing organs (Lee et al., 1995). In addition, col II-PTHrP/PTHrP-null mice have defects in other ectodermally derived organs (skin, teeth and sebaceous glands) that are dependent on epithelial-mesenchymal interactions for their development (unpublished observations). We anticipate that PTHrP will be found to participate in the regulation of mesenchymal cell function during the development of a number of epithelial organs, and it is our hope that further study of the effects of PTHrP during embryonic mammary development will provide a framework for the general understanding of PTHrP's role in regulating mesenchymal function during organogenesis.

We thank J. McCaughern-Carucci, J.P. Zhang and B. Dreyer for expert technical assistance. This work was supported by NIH grants CA60498, AR 30102, DK 31998, DOD grant DAMD17-96-1-6198, and a pilot project grant from the Yale Diabetes and Endocrine Research Center (NIH 5-P30-DK45735).

REFERENCES

Amling, M. Neff, L. Tanaka, S., Inoue, D., Kuide, K. Weir, E., Philbrick, W. M., Broadus, A. E. and Baron, R. (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J. Cell Biol.* **136**, 205-213.

Broadus, A. E. and Stewart, A. F. (1994). Parathyroid hormone-related protein: structure, processing and physiologic actions. In *The Parathyroids* (ed. J. P. Bilizekian, M. A. Levine and R. Marcus). New York: Raven Press.

Budayr, A. A., Halloran, B. R., King, J., Diep, D., Nissenson, R. A. and Strewler, G. J. (1989). High levels of a parathyroid hormone-related protein in milk. *Proc. Natl. Acad. Sci. USA* **86**, 7183-7185.

Cunha, G. R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74**, 1030-1044.

Cunha, G. R., Young, P., Christov, K., Guzman, R. Nandi, S., Talamantes, F. and Thordarson, G. (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* **152**, 195-204.

Freire-Maia, N., and Pinheiro, M. (1994). Ectodermal dysplasias: a clinical classification and causal review. *Am. J. Med. Genet.* **53**, 153-162.

Geise, K., Pagel, J. and Grosschedl, R. (1994). Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors. *Proc. Natl. Acad. Sci. USA* **91**, 3368-3372.

Henderson, J. E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B. M. K., Goltzman, D. and Karaplis, A. C. (1995). Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Molec. Cell. Biol.* **15**, 4064-4075.

Jüppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Hock, J., Potts, J. T., Kronenberg, H. M. and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254**, 1024-1026.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

Kopan, R. and Fuchs, E. (1989). A new look at an old problem: keratins as tools to investigate determination, morphogenesis and differentiation in skin. *Genes Dev.* **3**, 1-15.

Kovacs, C. S., Lanske, B., Hunzelman, J. L., Guo, J., Karaplis, A. C. and Kronenberg, H. M. (1996) Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proc. Natl. Acad. Sci. USA* **93**, 15233-15238.

Kratochwil, K. and Schwartz, P. (1976). Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue of testosterone. *Proc. Natl. Acad. Sci. USA* **73**, 4041-4044.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J. and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**, 1382-1394.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Jüppner, H., Segre, G. V. and Kronenberg, H. M. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science.* **273**, 663-666.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**, 453-463.

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., Kronenberg, H. M. and Segre, G. V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology* **137**, 5109-5118.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E. and Stewart, A.F. (1996) Defining the physiologic roles of parathyroid hormone-related protein in normal physiology. *Physiological Reviews* **76**, 127-173.

Phippard, D. B., Weber-hall, S. J., Sharpe, P. T., Naylor, M. S., Jayatalake, H., Maas, R., Woo, I., Roberts-Clarke, D., Francis-West, P. H., Liu, Y. H., Maxson, R., Hill, R. E. and Dale, T. C. (1996). Regulation of Msx-1, Msx-2, BMP-2 and BMP-4 during foetal and postnatal mammary gland development. *Development* **122**, 2729-2737.

Propper, A. (1973) Evolution en culture in vitro de l'epithelium mammaire d'embryon de lapin associe au mesoderme d'osieau. *C. R. Acad. Sci. Paris* **277**, 2409-2412.

Propper, A. and Gomot, L. (1973). Control of chick epidermis differentiation by rabbit mammary mesenchyme. *Experientia* **29**, 1543-1544.

Sakakura, T. (1987). Mammary embryogenesis. In *The Mammary Gland: Development, Regulation and Function.* (ed. M. C. Neville and C. W. Daniel) pp. 37-66. New York: Plenum Press.

Sakakura, T. (1991). New aspects of stroma-parenchyma relations in mammary gland differentiation. *Int. Rev. Cytology* **125**, 165-199.

Smith, G. H., Mehrel, T. and Roop, D. R. (1990). Differential keratin gene expression in developing, differentiating, and neoplastic mouse mammary epithelium. *Cell Growth Differ.* **1**, 161-170.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39**, 35-50.

Thiede, M. A. and Rodan, G.A. (1988). Expression of a calcium mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* **242**, 278-280.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R-G., Parslow, T. G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8**. 2691-2703.

Vortkamp, A., Lee, K., Lanske, B., Segre G. V., Kronenberg H. M. and Tabin, C. J. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Weil, M., Itin, A. and Keshet, E. (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121**, 2419-2428.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. E. and Broadus, A. E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondrol bone formation. *Proc. Natl. Acad. Sci. USA* **93**, 10240-10245.

Witty, J. P., Wright, J. H. and Matresian, L. M. (1995). Matrix mettaloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell* **6**, 1287-1303.

Wu, T., Vasavada, R., Yang, K., Massfelder, T., Ganz, M., Abbas, S. K., Care, A. D. and Stewart, A. F. (1996) Structural and physiologic characterization of the mid-region secretory form of PTHrP. *J. Biol. Chem.* **271**, 24371-24381.

Wysolmerski, J. J. and Broadus, A. E. (1994) Hypercalcemia of malignancy: the central role of parathyroid hormone related protein. *Annu. Rev. Med.* **45**, 189-200.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M. and Philbrick, W. M. (1994). Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl. Acad. Sci. USA* **91**, 1133-1137.

Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E. and Philbrick, W. M. (1995). Overexpression of parathyroid hormone related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary development. *Development* **121**, 3539-3547.

Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartman, G., Weidner, K. M., Birchmeier, C. and Birchmeier, W. (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131**, 215-226.

DEVELOPMENTAL BIOLOGY 203, 75–89 (1998)
ARTICLE NO. DB989029

Stromal Cells Are Critical Targets in the Regulation of Mammary Ductal Morphogenesis by Parathyroid Hormone-Related Protein

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Parathyroid hormone-related protein (PTHrP) was originally identified as the tumor product responsible for humoral hypercalcemia of malignancy. It is now known that PTHrP is produced by many normal tissues in which it appears to play a role as a developmental regulatory molecule. PTHrP is a normal product of mammary epithelial cells, and recent experiments in our laboratory have demonstrated that overexpression or underexpression of PTHrP in the murine mammary gland leads to severe disruptions in its development. The nature of these phenotypes suggests that PTHrP acts to modulate branching growth during mammary development by regulating mammary stromal cell function. We now demonstrate that throughout mammary development, during periods of active ductal-branching morphogenesis, PTHrP is produced by epithelial cells, whereas the PTH/PTHrP receptor is expressed on stromal cells. In addition, we show that mammary stromal cells in culture contain specific binding sites for amino terminal PTHrP and respond with an increase in intracellular cAMP. Finally, we demonstrate that the mammary mesenchyme must express the PTH/PTHrP receptor in order to support mammary epithelial cell morphogenesis. These results demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal signaling circuit that is necessary for mammary morphogenesis and that stromal cells are a critical target for PTHrP's action in the mammary gland. © 1998 Academic Press

Key Words: **mammary gland development; branching morphogenesis; mammary mesenchyme; mammary stroma; tissue recombination experiments.**

INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was initially discovered because of its pathogenic role in a common paraneoplastic syndrome known as humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). It derives its name from the fact that its gene and the parathyroid hormone (PTH) gene are both descended from a common ancestor through a process of gene duplication (Broadus and Stewart, 1994). As a result, the two genes share sequence homology and structural characteristics that allow amino-terminal species of PTH and PTHrP to signal through the same receptor, termed the type I PTH/PTHrP receptor (Jüppner *et al.*, 1991). Despite these similarities, these two peptides have evolved to serve very different functions. PTH is made solely by the parathyroid chief cells and is secreted into the systemic circulation where it functions as a classical peptide hormone regulating calcium homeostasis. In contrast, PTHrP is made by a wide variety of cell types, does not circulate, and appears to

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0012-1606/98 $25.00

function as a local autocrine or paracrine factor influencing cell growth and differentiation (Broadus and Stewart, 1994).

As noted above, amino-terminal PTHrP signals through a seven-transmembrane-spanning, G-protein-coupled receptor known as the type I PTH/PTHrP receptor (Jüppner *et al.*, 1991). This receptor subserves the calcium-regulating functions of PTH and PTHrP in classical PTH-target organs, but, like PTHrP, is also widely expressed in tissues not involved in calcium homeostasis (Orloff *et al.*, 1989). In these sites the receptor is often expressed in cells in close proximity to those expressing PTHrP, suggesting that it also mediates many of the normal physiologic functions of PTHrP (Lee *et al.*, 1995). However, it is also known that PTHrP is a polyprotein which, through a series of posttranslational processing steps, gives rise to several biologically active peptides not containing the amino terminus (Broadus and Stewart, 1994; Soifer *et al.*, 1992; Wu *et al.*, 1996). These peptides presumably signal through distinct, but as yet unidentified, receptors (Kovacs *et al.*, 1996; Orloff *et al.*, 1996).

PTHrP's role in mature organisms remains unclear, but in recent years evidence has accumulated to suggest that this peptide functions as an important developmental regulatory molecule (Dunbar *et al.*, 1996; Philbrick *et al.*, 1996). In fact, they have been reported to be one of the earliest peptide hormone/receptor pairs to be detected during mouse development and appear to participate in the formation of parietal endoderm (Behrendsten *et al.*, 1995; deStolpe *et al.*, 1993). Because PTHrP-knockout mice suffer from a fatal form of chondrodysplasia (Karaplis *et al.*, 1994), the most widely studied aspect of PTHrP's developmental functions has been its role in regulating chondrocyte differentiation during fetal bone development. In the absence of PTHrP, chondrocytes within the fetal growth plate appear to differentiate too rapidly, and the fetal bones ossify prematurely (Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). In contrast, mice overexpressing PTHrP in chondrocytes are born with a cartilaginous skeleton, resulting from a profound delay in chondrocyte differentiation (Weir *et al.*, 1996). Recent studies have demonstrated that PTHrP acts in a feedback loop with Indian Hedgehog and BMP's, in a pathway involved in controlling the rate at which immature chondrocytes progress through their program of differentiation (Kretzschmar *et al.*, 1997; Lanske *et al.*, 1996; Vortkamp *et al.*, 1996).

Another site in which PTHrP has clearly been shown to have an important developmental role is the mammary gland. Soon after its discovery, PTHrP was noted to be expressed in the pregnant and lactating mammary gland and to be present in large quantities in milk (Budayr *et al.*, 1989; Thiede and Rodan, 1988). Its function during pregnancy and lactation is still unknown but more recent data from transgenic models of PTHrP underexpression and overexpression have shown that it participates in the regulation of ductal branching morphogenesis during embryonic development as well as during sexual maturation and early pregnancy (Wysolmerski *et al.*, 1996; 1998). In the absence of PTHrP or the PTH/PTHrP receptor, mammary epithelial buds form, but fail to initiate ductal branching morphogenesis. Instead, the fetal epithelial cells degenerate and mammary glands do not form (Wysolmerski *et al.*, 1998). Overexpression of PTHrP or PTH within the mammary gland also perturbs ductal branching morphogenesis (Wysolmerski *et al.*, 1996). In this case, an excess of PTHrP results in severe defects in ductular proliferation and side branching during puberty and the inhibition of terminal ductule formation during early pregnancy. Hence, amino-terminal PTHrP, acting through the PTH/PTHrP receptor, appears to contribute to the regulation of ductal branching morphogenesis at several different stages of mammary development.

Like many other epithelial organs, the mammary gland is dependent on the sequential and reciprocal exchange of information between epithelial cells and neighboring mesenchymal cells for its proper morphogenesis (Cunha and Hom, 1996; Sakakura, 1991; Thesleff *et al.*, 1995). These epithelial–mesenchymal interactions are especially critical for the regulation of ductal branching morphogenesis. The studies cited above suggest that PTHrP might act to regulate this process during mammary development by serving as an epithelial signal acting to regulate mammary stromal cell function. In this study, we present data to support such a role for PTHrP. We demonstrate that during periods of active ductal branching morphogenesis, the PTHrP gene is expressed in the mammary epithelium, and the PTH/PTHrP receptor gene is expressed in mammary stroma. In addition, we show that mammary stromal cells bind and respond to amino-terminal PTHrP. Finally, we demonstrate that the presence of the PTH/PTHrP receptor in mammary mesenchyme is critical to the ability of the mesenchymal cells to support the initiation of ductal growth.

MATERIALS AND METHODS

In Situ Hybridization

In situ hybridization was performed on 5-μm paraffin sections as described previously (Wysolmerski *et al.*, 1998). Probes corresponded to a 349-bp genomic fragment of the mouse PTHrP gene and a 238-bp cDNA fragment of the PTH/PTHrP receptor gene (Weir *et al.*, 1996). Sense and antisense probes were generated from linearized fragments using an *in vitro* transcription kit (Promega, Madison, WI) in the presence of [^{35}S]UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 μg/ml in PBS for 17 min at room temperature) and acetylated with 0.25% acetic anhydride in the presence of 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 min. Sections were then rinsed in 2× SSC and incubated for 30 min in 0.66% *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) in 2× SSC, rinsed in 2× SSC, dehydrated in graded alcohol, treated with chloroform for 5 min, rehydrated, and then air-dried. The probes (1.5×10^7 cpm/ml) were then hybridized to the samples for 17 h at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 250 μg/ml tRNA,

100 μg/ml salmon sperm DNA, and 50 mM DTT. After hybridization, sections were rinsed in 1× SSC and washed twice in 2× SSC/50% formamide for 5 min at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 min. Following two rinses in 2× SSC, sections were again washed in 2× SSC/50% formamide at 52°C for 5 min, dehydrated through graded ethanol, air-dried, and dipped in a mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development of the emulsion, sections were counterstained with hematoxylin and mounted for microscopic examination.

Preparation of Mammary Stromal Cell Cultures

Mammary epithelial and stromal cells were isolated using a modification of a previously described procedure (Haslam and Levely, 1985). Briefly, the No. 4 inguinal mammary glands were dissected from 11- to 14-day pregnant CD-1 mice, minced with razor blades, and incubated overnight at 37°C in a digestion buffer containing DMEM/F12, 5% FBS, 0.2% dispase grade II, 0.2% collagenase type III, 50 μg/ml gentamycin, 100 units/ml nystatin, and 2.5 μg/ml amphoteracin B. Following digestion, the cells were pelleted by centrifugation at 1500 rpm, washed with DMEM, and then filtered through a 70-μm nitex mesh filter to remove mammary epithelial organoids. To enrich the remaining cells for mammary fibroblasts, we used the differential centrifugation method of Voyles and McGrath (1976). The flow-through following filtration was spun at 80*g* for 30 s to pellet epithelial cells, and the resulting supernatant, containing the mammary fibroblasts, was removed and plated in DMEM medium supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 units/ml nystatin, 2.5 μg/ml amphoteracin B, and 5 μg/ml each insulin and hydrocortisone. Finally, to remove any remaining epithelial cells, the medium was changed 4 h after plating, and the resulting cultures of mammary stromal cells were grown at 37°C in 5% CO_2 for 5 days.

To assess the purity of the mammary stromal cultures, we performed immunohistochemistry using anti-vimentin and anti-keratin 14 and anti-keratin 8,18 antibodies as stromal cell and epithelial cell markers, respectively. The anti-mouse vimentin antibody is a monoclonal antibody and was purchased from Boehringer Mannheim (Indianapolis). The K14 antibody is an affinity-purified rabbit polyclonal antibody and was a kind gift of Dr. D. Roop (Houston, TX). The anti-keratin 8,18 antibody is a mouse monoclonal and was purchased from Nova-Castra (Burlingame, CA). Immunohistochemistry was performed using standard techniques and primary antibody binding was detected using the Vector Elite avidin–biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3′-diaminobenzidine as a chromagen. Slides were counterstained using hematoxlyn.

RNA Isolation and RNase Protection Analysis

Total RNA was isolated from cells using Trizol reagent (Gibco, Gaithersville, MD). RNase protection analysis was performed as described previously (Daifotis *et al.*, 1992) using 2×10^5 cpm of labeled antisense cRNA probes corresponding to a 349-bp *Avr*II *Pvu*II genomic fragment of the mouse PTHrP gene and a 283-bp *Sau*3a–*Pvu*II fragment of the mouse PTH/PTHrP receptor gene. For an internal standard, 5×10^4 cpm of labeled antisense probe corresponding to a 220-bp *Sau*3a–*Sau*3a fragment of the mouse cyclophilin gene was used.

Receptor Binding Assay

The receptor binding assays were performed as described previously (Orloff *et al.*, 1992) using 30,000 cpm/well of ^{125}I-labeled [Tyr36]hPTHrP-(1–36) NH_2 in a final volume of 0.15 ml/well in 24-well plates for 4 h at 4°C in the presence or absence of increasing concentrations of competing unlabeled PTHrP(1–36).

cAMP Assay

Cells were grown to confluence in 12-well plates at 37°C in 5% CO_2. Prior to the experiment, the cells were washed once with serum-free medium containing 0.1% BSA and then incubated with serum-free medium/0.1% BSA with or without PTHrP (1–36) at various concentrations and for various time periods. The medium was then aspirated, and the cells were treated with ice cold 90% *n*-propanol for 24 h at −70°C. The samples were then lyophilized, and intracellular cAMP content was measured using a commercially available RIA (Biomedical Technologies, Stoughton, MA).

Tissue Recombination Experiments

Mammary gland rudiments were dissected from E13 PTH/PTHrP receptor knockout (ko) and wild-type (wt) embryos which were identified by their genotype as determined by PCR as described previously (Wysolmerski *et al.*, 1998). The mammary rudiments were incubated for 1.5 h in 1% Bacto-trypsin in calcium–magnesium-free Hanks' salt solution at 4°C. Following neutralization of the enzyme with 10% fetal bovine serum in DMEM, the epithelium and mesenchyme were teased apart with watchmakers forceps. For PTH/PTHrP receptor knockout embryos, the four possible tissue recombinants were prepared with mammary epithelium (MGE) and mammary mesenchyme (MGM) from wt and ko mice: wt-MGM + wt-MGE, wt-MGM + ko-MGE, ko-MGM + ko-MGE, and ko-MGM + wt-MGE (Cunha *et al.*, 1995). All tissue recombinants were transplanted beneath the renal capsule of female athymic nude mouse hosts (see web site http://mammary.nih.gov/tools/Cunha001/index.html for technical details). Following 1 month of growth, the grafts were harvested for histological analysis (Cunha *et al.*, 1995).

RESULTS

Expression of PTHrP and the PTH/PTHrP Receptor during Ductal Morphogenesis

To begin to study the mechanisms by which PTHrP and the PTH/PTHrP receptor regulate ductal growth and branching morphogenesis, we examined their temporal and spatial patterns of expression during fetal life, during sexual maturation, and during early to mid-pregnancy, three periods of active ductal growth during mammary development. We first sought to determine the temporal pattern of PTHrP and PTH/PTHrP receptor mRNA expression in the whole mammary gland by RNase protection analysis. Due to the small size of the mammary glands, this analysis was not possible for fetal time points and, therefore, we initiated these studies by examining the temporal pattern of PTHrP and PTH/PTHrP receptor expression in the preadolescent gland (3-week-old virgin), during puberty (6-week-old vir-

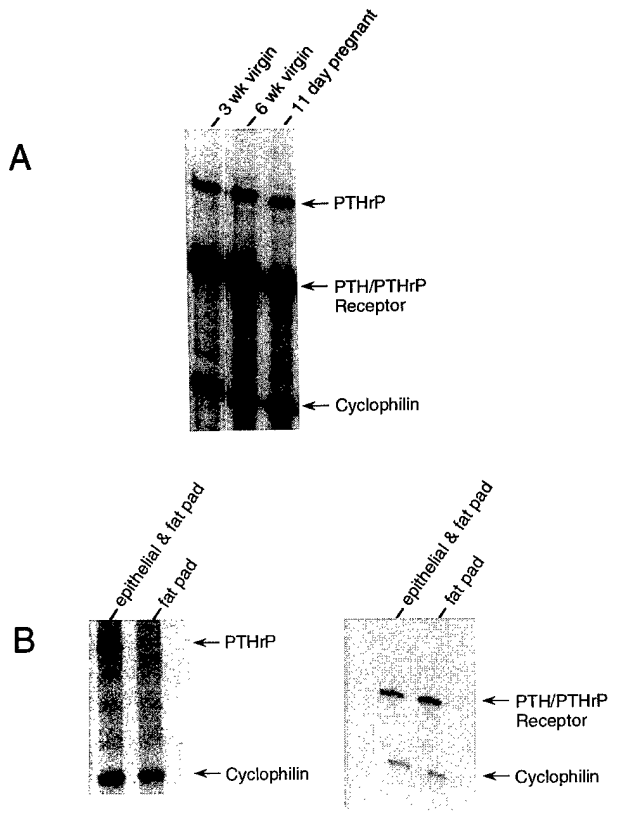

FIG. 1. (A) Analysis of PTH and PTH/PTHrP receptor RNA in the mammary gland during preadolescence, sexual maturation, and pregnancy. 50 μg of total cellular RNA prepared from mammary tissue from preadolescent (3-week-old virgin), adolescent (6-week-old virgin), and pregnant (11 days postcoitus) mice was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. The murine cyclophilin RNA was included as a loading control. Note that both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. (B) Analysis of PTHrP and PTH/PTHrP receptor mRNA expression in proximal and distal segments of preadolescent mammary glands. Mammary glands from 3-week-old virgin mice were dissected and separated into proximal and distal segments. Whole mount analysis confirmed that the proximal segment contained both epithelial and stromal components, while the distal segment contained only stroma (data not shown). 50 μg of total cellular RNA prepared from either the proximal component (epithelial + fat pad) or the distal component (fat pad) was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that the proximal component with both epithelial and stromal cells contains both PTHrP and the PTH/PTHrP receptor mRNA, but the distal component, with stromal cells alone, contains only the PTH/PTHrP receptor.

gin), and during early to mid-pregnancy (11 days postcoitus). As shown in Fig. 1A, both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. In addition, despite the dramatic changes in cellular composition of the mammary gland at these different time points, whole-gland levels of PTHrP and PTH/PTHrP receptor mRNA expression remained relatively constant.

Our prior studies had suggested that, during embryonic mammary development, PTHrP mRNA is expressed in mammary epithelial cells and PTH/PTHrP receptor mRNA is expressed in the mammary mesenchyme (Wysolmerski *et al.*, 1998). To determine if this pattern was also present during the later stages of ductal morphogenesis, we first took advantage of the directional growth of mammary epithelial ducts during puberty. Prior to the initiation of adolescence the mammary ducts occupy only a small portion of one end of the mammary fat pad and, in response to the hormonal stimulation of puberty, they grow toward the opposite end of the mammary fat pad until they completely fill out this stromal compartment. As a result of this growth pattern, at the initiation of puberty, one can divide the murine mammary gland into a proximal segment that contains both epithelial and stromal components and a distal segment that contains only stroma. As shown in Fig. 1B, the proximal component with both epithelial and stromal cells, contains both PTHrP and PTH/PTHrP receptor mRNA, but the distal component, that is stroma alone, contains only PTH/PTHrP receptor mRNA. These data suggest that PTHrP mRNA is expressed in the mammary epithelium, and that PTH/PTHrP receptor mRNA is expressed within the fat pad stroma.

To examine this possibility directly, we next determined the spatial localization of PTHrP and PTH/PTHrP receptor mRNA expression by *in situ* hybridization during fetal life, during adolescence and during early pregnancy. As shown in Fig. 2, the PTHrP gene was expressed in epithelial cells during periods of mammary ductal growth. At E12, we found PTHrP expression to be very intense in the epithelial cells of the embryonic mammary bud, especially in the cells located peripherally, adjacent to the basement membrane (Figs. 2A and 2B). At E18, at a time when the mammary bud is elongating and initiating ductal branching morphogenesis, PTHrP expression continued to be intense and was localized to mammary epithelial cells (Figs. 2C and 2D). Once again, expression of the PTHrP gene was most obvious in epithelial cells located on the outer most layer of the developing mammary ducts. These results are in agreement with our previous findings that demonstrated PTHrP mRNA expression in epithelial cells of the embryonic mammary bud at E16 (Wysolmerski *et al.*, 1998).

In the postnatal mammary gland (Figs. 2E through 2M), PTHrP mRNA expression continued to be localized to epithelial cells. However, overall, expression appeared to be less intense than during fetal development and it appeared to be restricted to epithelial cells located within terminal end buds. End buds are specialized structures that form at the leading edge of growing ducts, and they serve as the sites of active cellular proliferation and differentiation during phases of ductular proliferation (Daniel and Silberstein, 1987). As seen in Figs. 2E–2I, we found that, during puberty, PTHrP mRNA was present in the epithelial cells of end buds (Figs. 2E through 2G), but was undetectable in

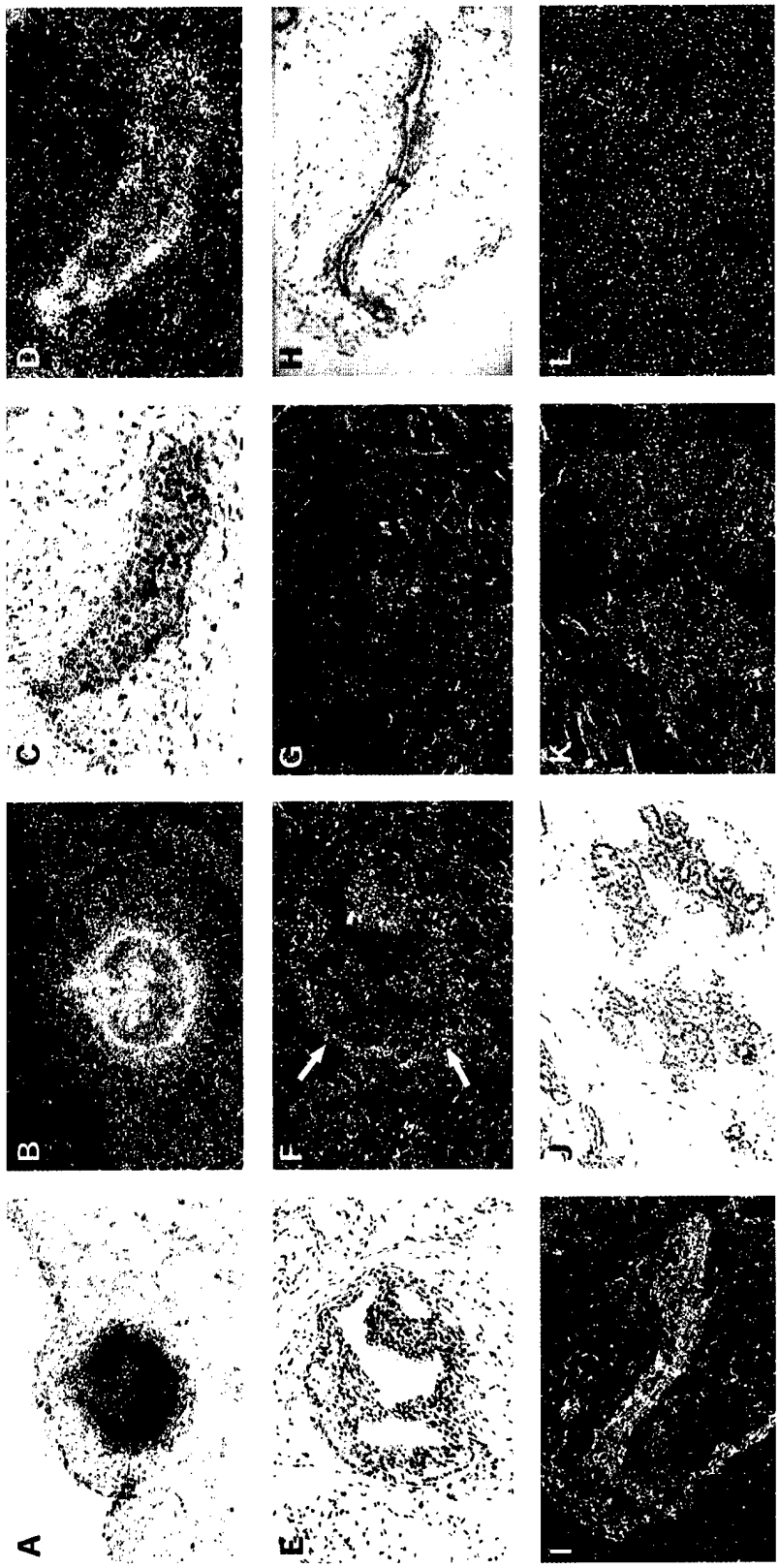

FIG. 2. Localization of PTHrP mRNA expression in mammary glands of embryonic, adolescent, and pregnant mice. (A–D) *In situ* hybridization for PTHrP mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images, respectively, of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTHrP mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of an adolescent mammary gland hybridized with an antisense probe. (J–M) *In situ* hybridization for PTHrP mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

epithelial cells of mature ducts (Figs. 2H and 2I). Specifically, the PTHrP mRNA signal appeared to localize to the peripheral, or cap cells of the end buds, a pattern similar to the peripheral location of the PTHrP signal seen during fetal life. During early pregnancy, there appeared to be a very low level of PTHrP mRNA expression within the epithelial cells of developing lobuloalveolar units (Figs. 2J through 2M) but, similar to puberty, we could not detect PTHrP mRNA in mature mammary ducts (data not shown). However, we did detect a similar pattern of epithelial PTHrP expression in the occasional end bud we observed at the periphery of early pregnant glands (data not shown). Therefore, it appears that similar to embryonic life, in the postnatal mammary gland, the major site of PTHrP expression is within epithelial cells. During puberty the PTHrP gene is expressed most prominently in end buds, and during early pregnancy there appears to be a low level of PTHrP expression within the developing lobuloalveolar units.

In contrast to the epithelial pattern of PTHrP mRNA expression, PTH/PTHrP receptor mRNA appeared to be expressed in mammary stromal cells (Fig. 3). In the embryonic mammary gland, at E12, PTH/PTHrP receptor mRNA was expressed throughout the ventral mesenchyme, including the dense mammary mesenchyme (Figs. 3A and 3B). At E18, when the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in stromal cells enveloping the growing mammary ducts (Figs. 3C and 3D). During puberty, PTH/PTHrP receptor mRNA was expressed at a low level throughout the mammary stroma, but the most prominent PTH/PTHrP receptor expression was in stromal cells immediately surrounding terminal end buds (Figs. 3E through 3G). This expression appeared to be most intense at the neck regions of the end buds and decreased rapidly along the more mature portions of the duct so that the majority of the periductal stroma demonstrated a level of receptor mRNA expression indistinguishable or just above the background expression of the fat pad stroma (Figs. 3H and 3I). During early to mid-pregnancy PTH/PTHrP receptor mRNA also appeared to be expressed at a low level throughout the fat pad stroma both within the periductal stroma and surrounding the developing lobuloalveolar units (Figs. 3J through 3M). However, the signal intensity was very low in the pregnant tissue, and there was not striking pattern of hybridization such as that seen during puberty. The exception to this diffuse low level of hybridization was in the occasional gland with a few remaining end buds during early pregnancy, where there was a strong signal within the stroma surrounding the end buds (not shown).

Together, these studies demonstrate that during active mammary ductular branching morphogenesis, PTHrP is expressed by epithelial cells, and its receptor is expressed by surrounding mesenchymal cells. In addition, it appears that in the postnatal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor gene is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty.

Mammary Stromal Cells Express Functional PTH/PTHrP Receptors

Our *in situ* hybridization results demonstrate that the PTH/PTHrP receptor is expressed in mammary stromal cells and, therefore, suggest that these cells are a target for PTHrP's action in the mammary gland. To test this hypothesis, we prepared primary cultures of both epithelial and stromal cells from adult female mammary glands using previously described protocols (Haslam and Levely, 1985; Voyels and McGrath, 1976) and characterized their composition by immunocytochemistry, using anti-vimentin and anti-keratin 14 and anti-keratin 8, 18 antibodies to identify fibroblasts and epithelial cells, respectively. As defined by the expression of vimentin and the lack of keratin expression, we were able to consistently prepare primary cultures of mouse mammary stromal cells that were 90–95% pure, a level of stromal cell enrichment comparable to that of previous reports (data not shown, Haslam and Levely, 1985).

To determine whether stromal cells in culture continued to express PTH/PTHrP receptor mRNA, total RNA was prepared from our stromal cell cultures and from freshly isolated mammary epithelial organoids and assayed for steady-state levels of both PTHrP and PTH/PTHrP receptor mRNA by RNase protection analysis. As shown in Fig. 4, mammary stromal cells in culture contained PTH/PTHrP receptor mRNA, but not PTHrP mRNA. In contrast, mammary epithelial cells in freshly isolated organoids contained PTHrP mRNA but no PTH/PTHrP receptor mRNA. These results are identical to the results from our *in situ* analysis and lend further support to the epithelial–mesenchymal pattern of expression of PTHrP and the PTH/PTHrP receptor in the mammary gland.

We next examined mammary stromal cells for specific binding sites for amino-terminal PTHrP. For this purpose, receptor binding assays were performed on intact cells, using ^{125}I-labeled PTHrP(1–36) as a ligand. These experiments documented specific binding of ^{125}I-labeled PTHrP-(1–36) amide to mammary stromal cells with an apparent K_d of 8.9 ± 1.4 nM. Binding was specific, as it was effectively competed with unlabeled PTHrP (1–36) amide (Fig. 5). The number of binding sites per cell, as determined by Scatchard analysis, was calculated to be 126,000 ± 13,000. In addition, treatment of mammary stromal cells with PTHrP(1–36) caused a increase in intracellular cAMP over basal (Fig. 6). This cAMP response peaked at 2 min, as shown in Fig. 6a, and showed a dose-dependent increase in cAMP accumulation with maximal responses occurring with 10^{-6} and 10^{-7} M PTHrP (Fig. 6b). Together, these results indicate that mouse mammary stromal cells express the PTH/PTHrP receptor both *in vivo* and *in vitro*, and cultured stromal cells display high-affinity binding sites for amino-terminal PTHrP at their cell surface and respond to PTHrP with an increase in intracellular cAMP.

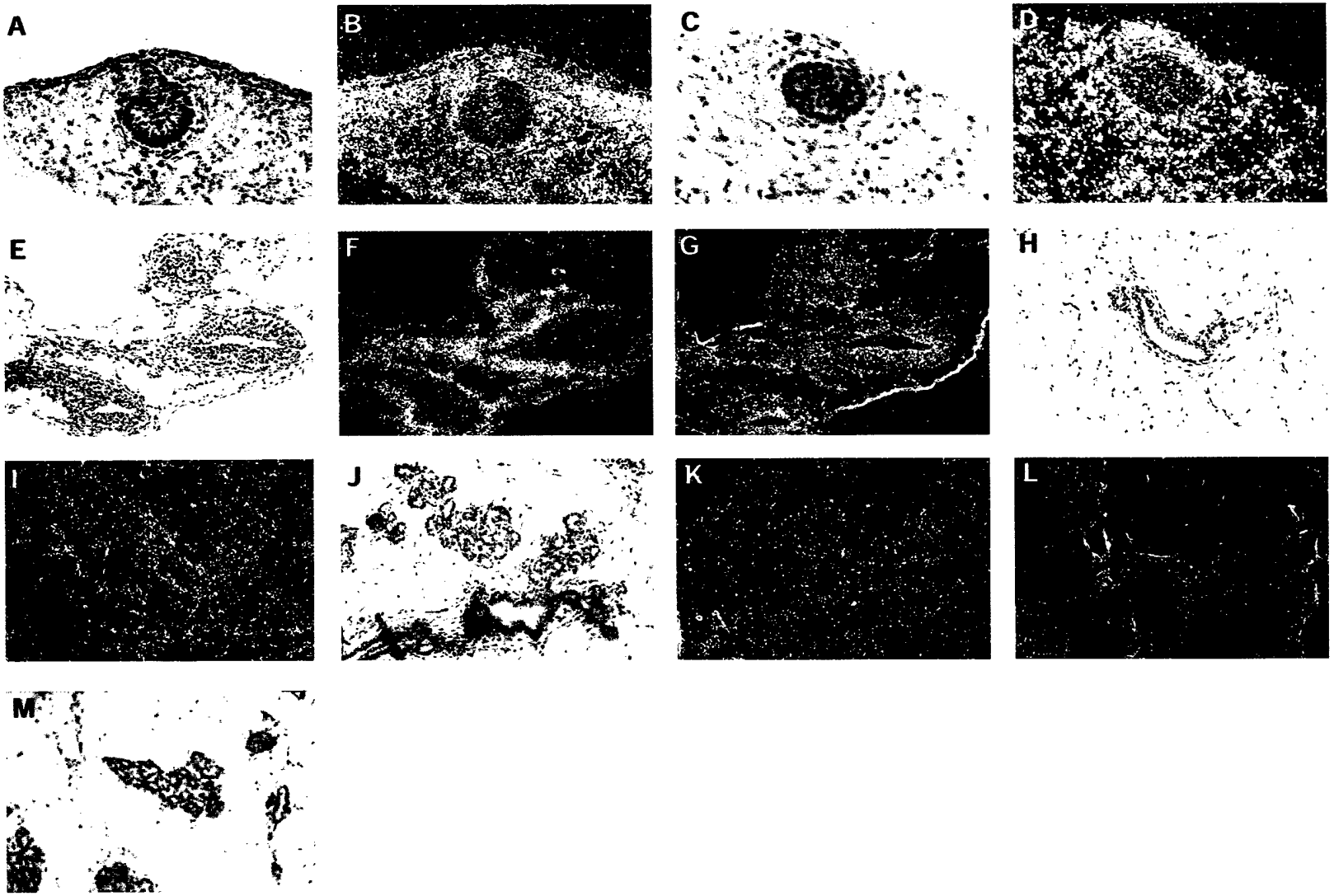

FIG. 3. Localization of PTH/PTHrP receptor mRNA during mammary development. *In situ* hybridization for PTH/PTHrP receptor mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTH/PTHrP receptor mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. (J–M) *In situ* hybridization for PTH/PTHrP receptor mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

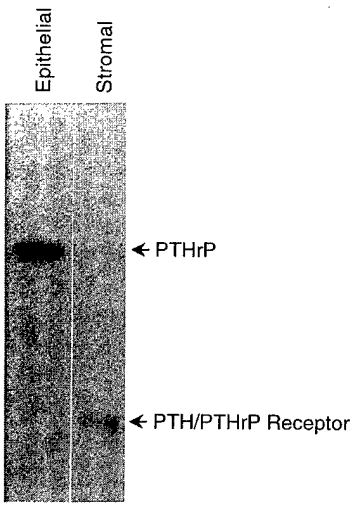

FIG. 4. Analysis of PTHrP and PTH/PTHrP receptor expression in freshly isolated mammary epithelial cells and mammary stromal cells in culture. 50 μg of total cellular RNA from freshly isolated mammary organoids and cultured mammary stromal cells was assayed for both PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that mammary stromal cells in culture express the PTH/PTHrP receptor but not PTHrP mRNA, whereas mammary epithelial cells express PTHrP mRNA but not PTH/PTHrP receptor mRNA.

The Mammary Mesenchyme Must Express the PTH/PTHrP Receptor to Support the Outgrowth of the Mammary Epithelium

We have previously reported that PTHrP and the PTH/PTHrP receptor are required for the outgrowth of the mammary epithelium during embryonic development. In mice lacking either PTHrP or the PTH/PTHrP receptor, mammary buds form but subsequent mammary development fails. In the absence of PTHrP or its receptor, mammary epithelial cells fail to undergo the initial stage of branching morphogenesis known as the primary growth spurt and, instead, degenerate and die (Wysolmerski *et al.*, 1998). These data, together with the expression patterns of PTHrP and the PTH/PTHrP receptor and the presence of functional PTH/PTHrP receptors in mammary stromal cells, as described above, suggest that epithelial-derived PTHrP, acting through stromal PTH/PTHrP receptors, plays an important role in regulating ductal morphogenesis during fetal life and perhaps also at later stages of development (Wysolmerski *et al.*, 1996; 1998). To test this hypothesis directly, we performed a series of tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild-type and PTH/PTHrP receptor-knockout embryos. In these experiments, knockout and wild-type mammary epithelial buds and mammary mesenchyme were recombined in the four possible combinations (see Table 1) and grown under the kidney capsule of recipient females. We reasoned that if the above hypothesis was valid, and PTHrP and the PTH/PTHrP receptor do represent an epithelial–mesenchymal signaling circuit, the PTH/PTHrP receptor-null phenotype would be expected to segregate with mesenchymal tissue. That is, receptor-knockout mesenchyme should be unable to support the outgrowth of either receptor-knockout or normal epithelial buds, but receptor-knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Table 1 and Fig. 7 summarize the results of these experiments. As expected, wild-type epithelial buds paired with wild-type mammary mesenchyme (wt-MGE + wt-MGM) consistently gave rise to a series of branched epithelial ducts contained within a fatty stroma (see Fig. 7A). In contrast, ductal outgrowth was never detected when PTH/PTHrP receptor-knockout epithelial buds were paired with PTH/PTHrP receptor-knockout mesencluyme (ko-MGE + ko-MGM, see Fig. 7B). These transplants gave rise to a fatty stroma that was devoid of mammary epithelial cells, reproducing the phenotype of the PTH/PTHrP receptor-knockout embryos. Recombinations consisting of PTH/PTHrP receptor-knockout epithelium paired with wild-type mesenchyme (ko-MGE + wt-MGM) uniformly gave rise to branched epithelial ducts within a fatty stroma (Fig. 7C). However, although the receptor-knockout epithelial buds consistently grew out and formed a rudimentary branching ductal structure, the growth of the resulting ducts appeared stunted compared with the ducts produced by wild-type epithelial buds paired with wild-type mesenchyme. Nonetheless, the PTH/PTHrP receptor-knockout epithelial cells survived and had the capacity to initiate branching morpho-

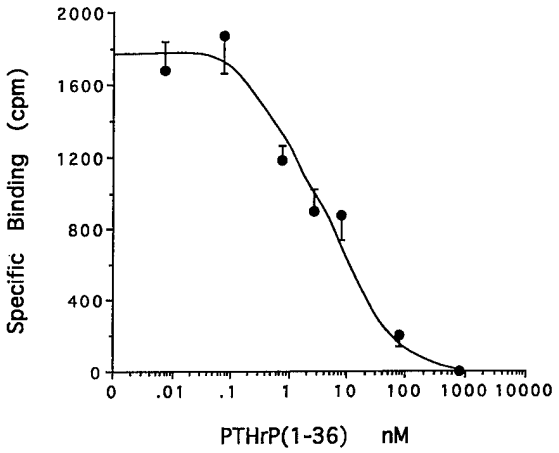

FIG. 5. Binding of PTHrP(1–36) to mammary stromal cells in culture. Receptor binding assays were performed with ^{125}I-labeled PTHrP(1–36) amide as a ligand for 4 h at 4°C with increasing concentrations of unlabeled PTHrP(1–36) as competitor. The data represent the mean ± SE. A representative of three independent experiments is shown.

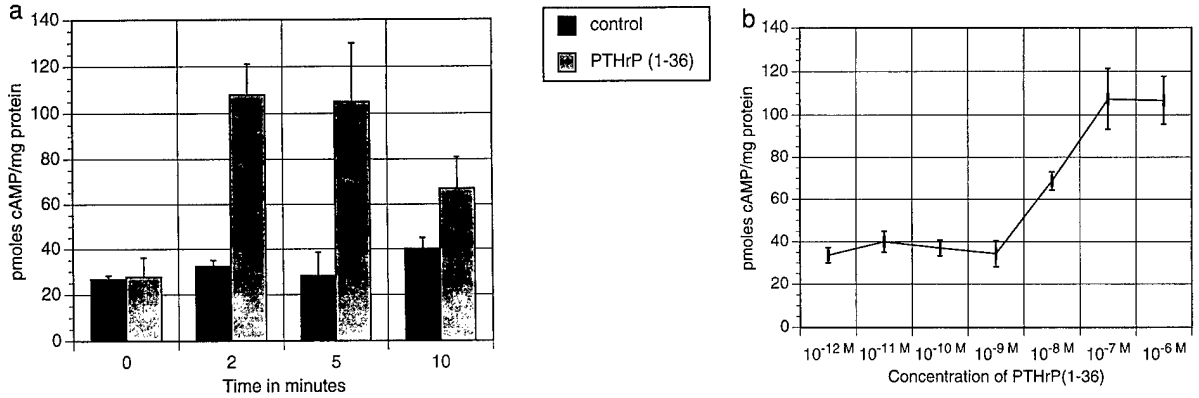

FIG. 6. cAMP response of mammary stromal cells in culture in response to PTHrP(1–36). (a) Time course of cAMP accumulation in primary cultures of mouse mammary stromal cells following treatment with PTHrP(1–36). Mammary stromal cells were incubated for the indicated times at 37°C in serum-free medium with or without 10^{-7} M PTHrP(1–36), and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE for duplicate samples from three separate experiments. (b) Dose-dependent effects of PTHrP(1–36) on cAMP accumulation in mammary stromal cells. Mammary stromal cells were treated with varying concentrations of PTHrP(1–36) for 2 min in serum-free medium, and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE of three experiments each run in duplicate.

genesis when paired with normal mesenchyme. As expected, similar to the results seen with knockout buds paired with knockout mesenchyme, all recombinants composed of wild-type epithelial buds paired with receptor-knockout mesenchyme (wt-MGE + ko-MGM) lacked any evidence of epithelial ductal outgrowth and consisted of fatty stroma alone (see Fig. 7D), suggesting that PTH/PTHrP receptor-knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. These results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PTH/PTHrP receptor null mice segregate with mesenchymal tissue and suggest that the mesenchyme is a critical target for the actions of PTHrP during mammary ductal morphogenesis.

DISCUSSION

Mammary gland morphogenesis is dependent on the interplay of systemic endocrine signals and short-ranged epithelial–mesenchymal interactions (Cunha, 1994; Sakakura, 1991). Although the morphological and endocrine aspects of mammary development have been well defined, the paracrine molecules and signaling mechanisms that are influenced by systemic hormones and that regulate mammary epithelial–mesenchymal interactions have only recently begun to be understood (Friedmann and Daniel, 1996; Kratochwil *et al.*, 1996; Phippard *et al.*, 1996; Hennighausen and Robinson, 1998). Mammary ductal growth during puberty is strictly dependent upon estrogens such as estradiol signaling through the estrogen receptor-α, a conclusion derived from the analysis of estrogen receptor-knockout (ERKO) mice (Korach, 1994). In addition, recent analysis of ERKO/wild-type tissue recombinants have shown that stromal estrogen receptors are especially important for the effects of estrogens on ductal growth and branching (Cunha *et al.*, 1997). Several stromal cell factors (some of which are estrogen-responsive) have now been identified that appear to regulate epithelial cell morphogenesis and differentiation (Alexander *et al.*, 1996; Jones *et al.*, 1996; Pollard and Henninghausen, 1994; Sympson *et al.*, 1994; Weil *et al.*, 1995; Witty *et al.*, 1995; Yang *et al.*, 1995). However, less is known about epithelial signals that are involved in regulating stromal cell function during mammary development. Prior experiments in transgenic mice have suggested that amino-terminal PTHrP, acting through the PTH/PTHrP receptor, might play a role in the epithelial–mesenchymal interactions that govern mammary ductal morphogenesis. In this report we provide a series of observations lending further support to the notion that PTHrP is an epithelial signal received by the mammary mesenchyme that is critical for the mesenchyme's ability

TABLE 1
Summary of Tissue Recombination Experiments

Mesenchyme	Epithelium	*n*	Stroma only	Ducts
wt	wt	4	0	4
Receptor-ko	Receptor-ko	4	4	0
wt	Receptor-ko	2	0	2
Receptor-ko	wt	3	3	0

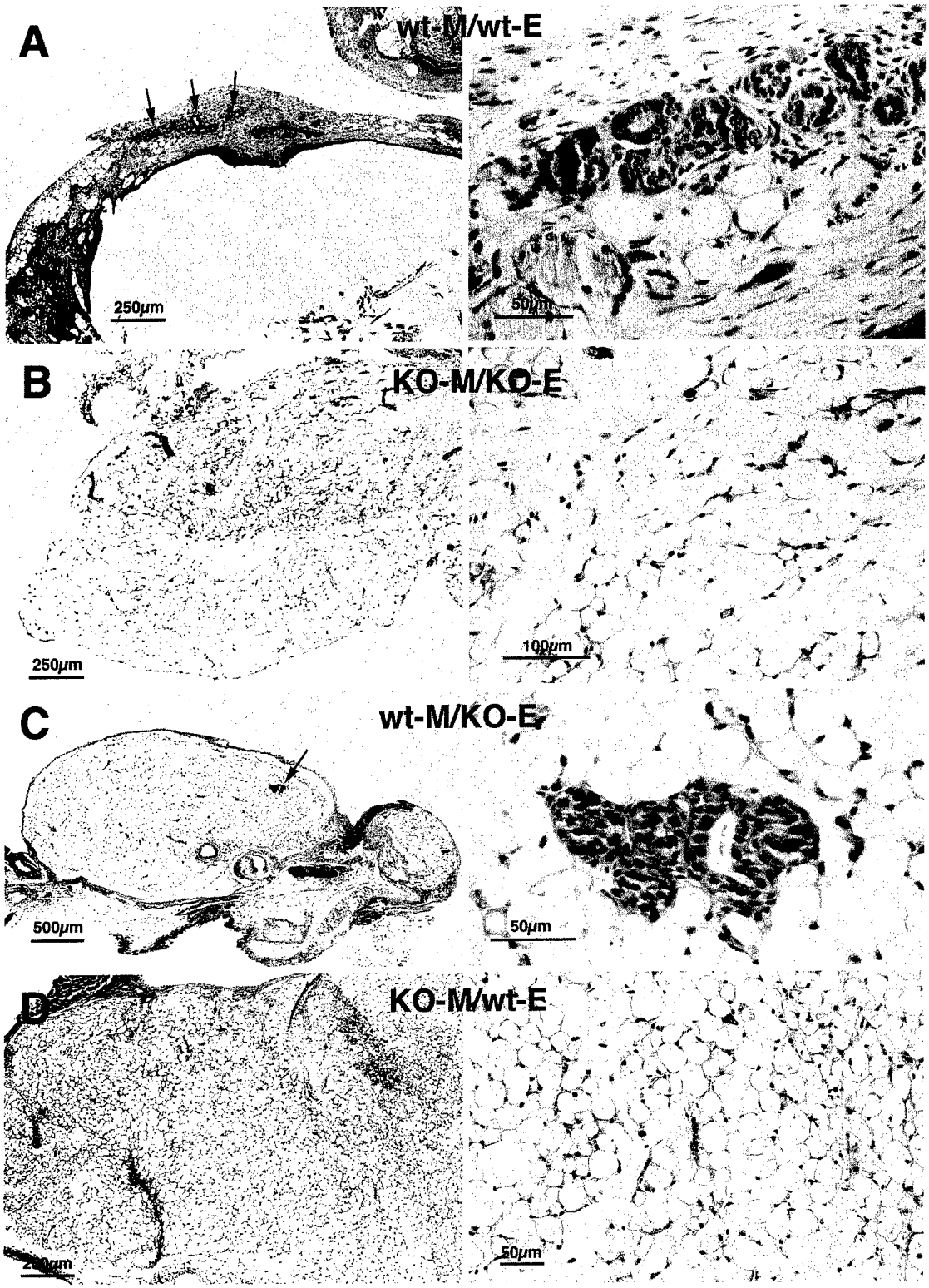

FIG. 7. Mesenchymal PTH/PTHrP receptor is necessary for the initiation of epithelial outgrowth. Mammary buds were dissected from wild-type and PTH/PTHrP receptor knockout embryos at E13, and the epithelium and mesenchyme were separated and then recombined in the four possible combinations and grown under the kidney capsule of recipient female mice for one month. Shown here are H&E-stained

to support epithelial cell outgrowth and ductal branching morphogenesis.

The first observation in support of this concept is that, during periods of active ductal morphogenesis, PTHrP and the PTH/PTHrP receptor are localized in epithelial and mesenchymal cells, respectively. Mammary ductal morphogenesis occurs in three distinct phases: during embryogenesis, during puberty and during early to mid-pregnancy. At each of these stages, we have shown that PTHrP is expressed in mammary epithelial cells and that the PTH/PTHrP receptor is expressed in mammary stromal cells. During embryogenesis, PTHrP mRNA was localized by *in situ* hybridization to the epithelial cells of the mammary bud and to the epithelial cells of rudimentary ducts as they progressed through the initial round of ductal branching morphogenesis from E16 to birth. The PTH/PTHrP receptor gene was expressed in the mesenchymal cells surrounding the mammary bud and in the mesenchyme that becomes associated with the branching epithelial ducts as they grow out into the developing mammary fat pad. This pattern of epithelial/mesenchymal expression was also observed during puberty. RNase protection analysis of PTHrP and PTH/PTHrP receptor expression demonstrated that PTHrP is found only within the portions of the mammary gland containing epithelial cells, but that PTH/PTHrP receptor mRNA is found both within the portions of the gland containing epithelium as well as within the fat pad alone. *In situ* hybridization demonstrated that this is due to the expression of the PTHrP gene in epithelial cells and its receptor gene in stromal cells. Furthermore, our *in situ* results demonstrated that, during puberty, both genes are most prominently expressed in terminal end buds. Interestingly, reminiscent of the fetal pattern of expression, we found that PTHrP mRNA appears to be localized to peripheral epithelial cells within the end buds which are known as cap cells (Daniel and Silberstein, 1987). PTH/PTHrP receptor message is found in the stromal cells immediately adjacent to the cap cells and enveloping the terminal end buds. Whereas we observed a low level of expression of the PTH/PTHrP receptor gene in the stroma of the fat pad and surrounding more mature ducts, we could not detect PTHrP mRNA within the epithelial cells of the mature ducts.

During pregnancy there was a low level of PTHrP mRNA expression in epithelial cells of the developing lobuloalveolar units, and it appeared that the PTH/PTHrP receptor gene was expressed throughout the stroma. These results are consistent with the findings of Rakopolous *et al.* (1992) who reported a diffuse, low level of PTHrP gene expression within mammary epithelial cells during pregnancy in rats. However, in our experiments, the levels of expression of both genes were just above the detection limit of our *in situ* hybridization technique and neither gene displayed an obvious pattern of expression as seen within the end buds of the adolescent gland. Despite this low level of hybridization, we are confident that PTHrP is expressed in epithelial cells and the PTH/PTHrP receptor is expressed in stromal cells during pregnancy for we obtained exactly these results using the more sensitive RNase protection assay. First, it is clear from Fig. 1A that both genes continue to be expressed on a whole gland level during pregnancy and, second, our analysis of epithelial and stromal cells isolated from pregnant mammary glands (Fig. 4) demonstrates PTHrP but no PTH/PTHrP receptor mRNA expression in epithelial cells and PTH/PTHrP receptor but not PTHrP mRNA in stromal cells.

The expression of PTHrP and its receptor in end buds in an epithelial/mesenchymal pattern is logical if PTHrP affects stromal function in a way that is important to the overall regulation of morphogenesis because the terminal end buds are the sites of active ductal growth and morphogenesis. During the embryonic development of the mammary gland, the initial round of branching morphogenesis occurs as a consequence of signaling between the mammary epithelial bud and its surrounding mesenchyme (Sakakura, 1991). Likewise, during puberty, epithelial–mesenchymal signaling at the terminal end bud influences the overall rate of ductal proliferation as well as the branching pattern of the growing duct system (Daniel and Silberstein, 1987; Silberstein and Daniel, 1987; Silberstein *et al.*, 1990). Therefore the recapitulation of the embryonic pattern of PTHrP and PTH/PTHrP receptor expression within the end buds suggests that PTHrP signaling to mesenchymal cells is most likely important to these processes during both phases of mammary gland development. This concept is supported

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sections through the resultant transplants after they were removed from beneath the kidney capsule. The left side of the figure represents low-power views and the right side represents the high-power magnifications of the same sections displayed on the left. Each transplant consists of a fragment of connective tissue containing varying amounts of fatty stroma, fibrous stroma, epidermal structures, and mammary epithelium. (A) Representative transplant resulting from wild-type epithelium recombined with wild-type mesenchyme (wt-MGE + wt-MGM). Note the mammary epithelial ducts (higher power view on right) located within a mixture of fibrous and fatty stroma. The structures at the lower left in the left-sided panel are hair follicles. (B) Representative transplant resulting from PTH/PTHrP receptor-knockout epithelium (ko-MGE) paired with PTH/PTHrP receptor-knockout mesenchyme (ko-MGM). Note that there are no epithelial ducts in this section, only fatty stroma. (C) Representative transplant resulting from wild-type mesenchyme (wt-MGM) paired with PTH/PTHrP receptor-knockout epithelium (ko-MGE). Note that epithelial ducts are present within the stroma (higher magnification on right), but that there are fewer ducts than in A. (D) Representative transplant resulting from knockout mesenchyme (ko-MGM) paired with wild-type epithelium (wt-MGE). As in B, note the complete absence of epithelial ducts. The arrows in A and C indicate mammary epithelial ducts. The scale bars in each panel demonstrate magnification as labeled.

by the results of our experiments in transgenic animals because disruption of the PTHrP gene results in the failure of the initial round of branching outgrowth during embryonic development and overexpression of PTHrP in the mammary gland in transgenic mice results in defects in ductular proliferation and branching during adolescence and pregnancy (Wysolmerski *et al.*, 1996; 1998).

We did not detect PTH/PTHrP receptor expression in epithelial cells by *in situ* hybridization during the time points we examined. This is in contrast to reports in the literature that have demonstrated PTH/PTHrP receptor expression in cultured myoepithelial cells and in several breast cancer cell lines (Birch *et al.*, 1995; Ferrari *et al.*, 1993; Seitz *et al.*, 1993). It may be that our *in situ* hybridization techniques cannot detect low levels of PTH/PTHrP receptor mRNA in myoepithelial cells, or perhaps the timing of PTH/PTHrP receptor expression in epithelial cells is not represented in our sampling. Alternatively, the presence of the receptor in myoepithelial cells may be specific to cultured or transformed cells. Our results cannot exclude that there may also be effects of PTHrP directly on some epithelial cells. However, these results clearly do demonstrate that the major location of PTH/PTHrP receptor expression is the stroma.

The second observation in support of stromal cells as a target of PTHrP's effects in the mammary gland is the ability of cultured stromal cells to express the PTH/PTHrP receptor, to bind amino-terminal PTHrP with high affinity, and to generate cAMP in response to PTHrP. These results, combined with our findings that freshly isolated mammary epithelial cells express the PTHrP gene but not the PTH/PTHrP receptor gene, represent a correlation of the *in situ* findings, *in vitro,* and underscore the concept that PTHrP, produced by epithelial cells, acts on stromal cells. This pattern is probably not unique to the mammary gland because dermal fibroblasts and lung fibroblasts have also been shown to respond to PTH and PTHrP, presumably via the PTH/PTHrP receptor (Rubin *et al.*, 1994; Shin *et al.*, 1997; Wu *et al.*, 1987). Furthermore, the ability of these cultured cells to retain their response to PTHrP now offers us an experimental system with which to begin to study the biological responses of stromal cells to PTHrP.

Our final observation in support of our working hypothesis is the demonstration that the expression of the PTH/PTHrP receptor in the mesenchyme is necessary for mammary epithelial cell survival and ductal morphogenesis. Using heterotypic tissue recombination and transplantation experiments, we have demonstrated that mammary mesenchymal cells require functional PTHrP signaling for these cells to support the survival and outgrowth of embryonic mammary epithelial cells. Unfortunately, experiments using tissues from PTHrP knockout embryos were uninformative, but mesenchymal cells taken from PTH/PTHrP receptor-knockout embryos were not able to support the survival or growth of either receptor-knockout or wild-type epithelial cells when these tissues were transplanted under the kidney capsule of recipient mice. These results mirror the failure of PTH/PTHrP receptor-knockout mammary buds to undergo the initial, embryonic round of ductal branching morphogenesis *in vivo* (Wysolmerski *et al.*, 1998). However, receptor-knockout epithelial cells were able to survive and initiate branching morphogenesis when recombined with normal mesenchyme, demonstrating directly that mesenchymal cells are critical targets of PTHrP's actions in promoting the outgrowth of the mammary epithelial bud. It should be noted that although receptor-knockout epithelium grew out and formed a rudimentary duct system when combined with normal mesenchyme, the growth of the resultant epithelial ducts was clearly not normal. These results may reflect the possibility that myoepithelial cells express a low level of PTH/PTHrP receptor which is below the detection limit of our *in situ* hybridizations (discussed above) but which is, nonetheless, important for the subsequent growth of the epithelial ducts following the initiation of branching morphogenesis. It may be that PTHrP signaling to the mesenchyme is sufficient to initiate branching growth during fetal life but that subsequent ductular proliferation requires a more complicated signaling cascade involving PTHrP's actions on both stromal and myoepithelial cells. To clarify this issue as well as the temporal requirements for PTHrP during ductular morphogenesis, it is likely that we will need to employ more sensitive localization techniques and to generate either conditional PTHrP-overexpressing or conditional PTHrP-knockout mice.

Despite the caveats noted in the previous paragraph, in the aggregate, it is clear that PTHrP's actions on mammary stromal cells have important consequences for ductal morphogenesis. Much work has demonstrated that the stromal mesenchyme plays a key role in determining the morphology of the epithelial duct system as well as in regulating the differentiation and functional activity of the mammary epithelium (Cunha *et al.*, 1995; Propper, 1973; Propper and Gomot, 1973). Recent experiments have suggested that the stromal regulation of epithelial function is complex. The stroma secretes growth factors such as KGF, HGF/SF, IGF1, and neuregulin which have been shown to regulate ductal morphogenesis (Hadsell *et al.*, 1996; Niranjan *et al.*, 1995; Ulich *et al.*, 1994; Yang *et al.*, 1995). In addition, the stroma contributes to the extracellular matrix, whose composition can have profound influences on epithelial behavior (Bissell and Hall, 1987; Sakakura, 1991). Finally, the stroma appears to be the principal source of matrix remodeling enzymes that also have been shown to have important effects on epithelial cell form, function, and survival (Alexander *et al.*, 1996; Sympson *et al.*, 1994; Witty *et al.*, 1995). All of these molecules are potential downstream stromal effectors of PTHrP's actions on epithelial development, and we are currently examining PTHrP's effects on their expression in our cultured mammary stromal cells. However, irrespective of the exact stromal response to PTHrP, our results underscore the truly reciprocal nature of the epithelial–mesenchymal interactions at play during the regulation of epithelial morphogenesis. Although stromal cells and their

products are critical to the regulation of epithelial form and function, it is clear that the epithelial cells participate in the regulation of their own fate, for without epithelial signals, such as PTHrP, the stromal cells are incompetent to direct epithelial morphogenesis.

In summary, our experiments demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal circuit that is necessary for mammary morphogenesis. Specifically, PTHrP produced by mammary epithelial cells must signal through the PTH/PTHrP receptor in mammary mesenchymal cells in order for the mesenchyme to support the initiation of mammary ductal morphogenesis. PTHrP has also been implicated in the development of other tissues that rely on epithelial/mesenchymal interactions for their development, including lung, teeth, and hair follicles (Philbrick *et al.*, 1998). Therefore, understanding PTHrP's role in regulating stromal cell function during the epithelial/mesenchymal interactions that govern mammary development should allow us to better understand the overall role of PTHrP in development.

ACKNOWLEDGMENTS

We thank Drs. Arthur Broadus and William Philbrick for their critical reading of the manuscript. We are grateful for the excellent technical assistance of Lina Golovyan. This work was supported by the DOD Grant DAMD17-96-1-6198 (J.J.W.) and NIH Grants CA 60498 (J.J.W.), CA 62114 (J.J.O.), CA 58207 (G.C.), and P01CA44768 (G.C.). M.E.D. is supported by the DOD Postdoctoral Fellowship DAMD17-97-1-7137. We are grateful for the use of the microscopy facilities of the Cell Biology Core of the Yale Diabetes and Endocrine Research Center.

REFERENCES

Alexander, C. M., Howard, E. W., Sympson, C. J., Bissell, M. J., and Werb, Z. (1996). Rescue of stromelysin-1-induced mammary epithelial cell apoptosis and entactin degradation by a TIMP-1 transgene. *J. Cell Biol.* **135,** 1669–1677.

Amizuka, N., Warshawasky, H., Henderson, J. E., Goltzman, D., and Karaplis, A. C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol.* **126,** 1611–1623.

Behrendsten, O., Alexander, C. M., and Werb, Z. (1995). Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos. *Development* **121,** 4137–4148.

Birch, M. A., Carron, J. A., Scott, M., and Gallagher, J. A. (1995). Parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor expression and mitogenic responses in human breast cancer cell lines. *Br. J. Cancer* **72,** 90–95.

Bissell, M. J., and Hall, H. G. (1987). Form and function in the mammary gland: The role of the extracellular matrix. *In* "The Mammary Gland: Development, Regulation, and Function (M. C. Neville and C. W. Daniel, Eds.). Plenum, New York.

Broadus, A. E., and Stewart, A. F. (1994). Parathyroid hormone-related protein: Structure, processing and physiologic actions. *In* "The Parathyroids" (J. P. Bilizekian, M. A. Levine, and R. Marcus, Eds.). Raven Press, New York.

Budayr, A. A., Holloran, B. R., King, J., Diep, D., Nissenson, R. A., and Strewler, G. J. (1989). High levels of parathyroid hormone-related protein in milk. *Proc. Natl. Acad. Sci. USA* **86,** 7183–7185.

Cunha, G. R. (1994). Role of mesenchymal–epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74,** 1030–1044.

Cunha, G. R., and Hom, Y. H. (1996). Role of mesenchymal–epithelial interactions in mammary gland development. *J. Mammary Gland Biol. Neoplasia* **1,** 21–35.

Cunha, G. R., Young, P., Christov, K., Guzman, F., Nandi, S., Talamantes, F., and Thordarson, G. (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* **152,** 195–204.

Cunha, G. R., Young, P., Hom, Y. K., Cooke, P. S., Taylor, J. A., and Lubahn, D. B. (1997). Elucidation of a role of stromal steroid hormone receptors in mammary gland growth and development by tissue recombination experiments. *J. Mammary Gland Biol. Neoplasia* **2,** 293–402.

Daifotis, A. G., Weir, E. C., Dreyer, B. E., and Broadus, A. E. (1992). Stretch induced parathyroid hormone related peptide gene expression in the rat uterus. *J. Biol. Chem.* **267,** 23455–23458.

Daniel, C. W., and Silberstein, G. B. (1987). Postnatal development of the rodent mammary gland. *In* "The Mammary Gland: Development, Regulation, and Function" (M. C. Neville and C. W. Daniel, Eds.), pp. 3–31. Plenum, New York.

deStolpe, A., Karperian, M., Lowik, C., Jüppner, H., Segre, G., and Abou-Samra, A. (1993). Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell Biol.* **120,** 235–243.

Dunbar, M., Wysolmerski, J. J., and Broadus, A. E. (1996). Parathyroid hormone-related protein: From humoral hypercalcemia of malignancy to developmental regulatory molecule. *Am. J. Med. Sci.* **312,** 287–294.

Ferrari, S., Rizzoli, R., Chaponnier, C., Gabbiani, G., and Bonjour, J.-P. (1993). Parathyroid hormone-related protein increases cAMP production in mammary epithelial cells. *Am. J. Physiol.* **264,** E471–E475.

Friedmann, F., and Daniel, C. W. (1996). Regulated expression of homeobox genes Msx-1 and Msx-2 in mouse mammary gland development suggests a role in hormone action and epithelial–stromal interactions. *Dev. Biol.* **177,** 347–355.

Hadsell, D. L., Greenberg, N. M., Fligger, J. M., Baumrucker, C. R., and Rosen, J. M. (1996). Targeted expression of des(1-3) human insulin-like growth factors I in transgenic mice influences mammary gland development and IGF-binding protein expression. *Endocrinology* **137,** 321–330.

Haslam, S. Z., and Levely, M. L. (1985). Estrogen responsiveness of normal mouse mammary cells in primary cell culture: Association of mammary fibroblasts with estrogen regulation of progesterone receptors. *Endocrinology* **116,** 1835–1844.

Henninghausen, L., and Robinson, G. W. (1998). Think globally, act locally: The making of a mouse mammary gland. *Genes Dev.* **12,** 449–455.

Jones, F. E., Jerry, D. J., Guraino, B. C., Andrews, G. C., and Stern, D. F. (1996). Heregulin induces in vivo proliferation and differentiation of mammary epithelium into secretory lobuloalveoli. *Cell Growth Differ.* **7,** 1031–1038.

Jüppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Hock, J., Potts, J. T., Kronenberg, H. M., and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related protein. *Science* **254,** 1024–1026.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M., and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of parathyroid hormone-related peptide gene. *Genes Dev.* **8,** 277–289.

Korach, K. (1994). Insights from the study of animals lacking functional estrogen receptor. *Science* **266,** 1524–1527.

Kovacs, C., Lanske, B., Hunzelman, J., Guo, J., Karaplis, A., and Kronenberg, H. (1996). Parathyroid hormone related peptide (PTHrP) regulates fetal–placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proc. Natl. Acad. Sci. USA* **93,** 15233–15238.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J., and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10,** 1382–1394.

Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massague, J. (1997). The TGF-beta family mediator Smad1 is phosphorylated and activated functionally by the BMP receptor kinase. *Genes Dev.* **11,** 984–995.

Lanske, B., Karaplis, A., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L., Ho, C., Mulligan, R., Abou-Samra, A., Jüppner, H., Segre, G., and Kronenberg, H. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-related bone growth. *Science* **273,** 663–666.

Lee, K., Deeds, J., and Segre, G. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136,** 453–463.

Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. (1995). HGF/SF: A potent cytokine for mammary growth, morphogenesis and development. *Development* **121,** 2897–2908.

Orloff, J. J., Wu, T. L., and Stewart, A. f. (1989). Parathyroid hormone-like proteins: Biochemical responses and receptor interactions. *Endocr. Rev.* **10,** 476–495.

Orloff, J. J., Ganz, M. B., Ribaudo, A. E., Burtis, W. J., Reiss, M., Milestone, L. M., and Stewart, A. F. (1992). Analysis of parathyroid hormone related protein binding and signal transduction mechanisms in benign and malignant squamous cells. *Am J. Physiol.* **262,** E599–E607.

Orloff, J. J., Ganz, M. B., Nathanson, M. H., Moyer, M. S., Kats, Y., Mitnick, M., Behal, A., Gasalla-Herraiz, J., and Isales, C. M. (1996). A midregion parathyroid hormone-related peptide mobilizes cytosolic calcium and stimulates formation of inositol trisphosphate in a squamous carcinoma cell line. *Endocrinology* **137,** 5376–5385.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E., and Stewart, A. F. (1996). Defining the roles of parathyroid hormone related protein in normal physiology. *Physiol. Rev.* **76,** 127–173.

Philbrick, W. M., Dreyer, B. E., Nakchbandi, I. A., and Karaplis, A. C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc. Natl. Acad. Sci. USA* in press.

Phippard, D. B., Weber-Hall, S. J., Sharpe, P. T., Naylor, M. S., Jayatalake, H., Mass, R., Woo, I., Roberts-Clarke, D., Francis-West, P. H., Liu, Y. H., Maxson, R., Hill, R. E., and Dale, T. C. (1996). Regulation of Msx-1, Msx-2, BMP-2 and BMP-4 during foetal and postnatal mammary gland development. *Development* **122,** 2729–2737.

Pollard, J. W., and Henninghausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc. Natl. Acad. Sci. USA* **91,** 9312–9316.

Propper, A. (1973). Evolution en culture in vitro de l'epithlium mammaire d'embryon de lapin associe au mesoderme d'osieau. *CR Acad. Sci. Paris* **277,** 2409–2412.

Propper, A., and Gomot, L. (1973). Control of chick epidermis differentiation by rabbit mammary mesenchyme. *Experientia* **29,** 1543–1544.

Rakopolous, M., Vargas, S. J., Gillespie, M. T., Ho, P. W. M., Diefenbach-Jagger, H., Leaver, D. D., Grill, V., Moseley, J. M., Danks, J. A., and Martin, T. J. (1992). Production of parathyroid hormone-related protein by the rat mammary gland in pregnancy and lactation. *Am. J. Physiol.* **263,** E1077–E1085.

Rubin, L. P., Kifor, O., Hua, J., Brown, E. M., and Torday, J. S. (1994). Parathyroid hormone (PTH) and PTH-related protein stimulate surfactant phospholipid synthesis in rat fetal lung, apparently by a mesenchymal–epithelial mechanism. *Biochim. Biophys. Acta* **1223,** 91–100.

Sakakura, T. (1991). New aspects of stroma–parenchyma relations in mammary gland differentiation. *Int. Rev. Cytol.* **125,** 165–199.

Seitz, P. K., Cooper, K. M., Ives, K. L., Ishizuka, J., Townsend, C. M., Rajsraman, S., and Cooper, C. W. (1993). Parathyroid hormone-related protein production and action in a myoepithelial cell line derived from normal human breast. *Endocrinology* **133,** 1116–1124.

Shin, J. H., Changhua, J., Casinghino, S., McCarthy, T. L., and Centrella, M. (1997). Parathyroid hormone related protein enhances insulin-like growth factor-1 expression by fetal rat dermal fibroblasts. *J. Biol. Chem.* **272,** 23498–23502.

Silberstein, G. B., and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-beta. *Science* **237,** 291–293.

Silberstein, G. B., Strickland, P., Coleman, S., and Daniel, C. W. (1990). Epithelium-dependent extracellular matrix synthesis in transforming growth factor-beta 1-growth-inhibited mouse mammary gland. *J. Cell Biol.* **110,** 2209–2219.

Soifer, N., Dee, K., Insogna, K., Burtis, W., Matovcik, L., Wu, T., and Stewart, A. (1992). Secretion of a novel mid-region fragment of parathyroid hormone related protein by three different cell lines in culture. *J. Biol. Chem.* **267,** 18236–18243.

Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J., and Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J. Cell Biol.* **125,** 681–693.

Thesleff, I., Vaahtokari, A., Kettunen, P., and Aberg, T. (1995). Epithelial–mesenchymal signaling during tooth development. *Connect. Tissue Res.* **32,** 9–15.

Thiede, M., and Rodan, G. (1988). Expression of a calcium mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* **242,** 278–280.

Ulich, T. R., Cardiff, R., Yi, S., Bikhazi, N., Blitz, R., Morris, C. F., and Pierce, G. F., (1994). Keratinocyte growth factor is a growth factor for mammary epithelium in vivo. The mammary epithelium of lactating rats is resistant to the proliferative action of keratinocyte growth factor. *Am. J. Pathol.* **144,** 862–868.

Vortkamp, A., Lee, K., Lanske, B., Segre, G., Kronenberg, H., and Tabin, C. (1996). Regulation of the rate of cartilage differentia-

tion by Indian Hedgehog and PTH-related protein. *Science* **273,** 613–622.

Voyels, B. A., and McGrath, C. M. (1976). Markers to distinguish normal and neoplastic mammary epithelial cells in vitro: Comparison of saturation density, morphology and concanavalin A reactivity. *Int. J. Cancer* **18,** 498–501.

Weil, M., Itin, A., and Keshet, E. (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121,** 2419–2428.

Weir, E., Philbrick, W., Amling, M., Neff, L., Baron, R., and Broadus, A. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc. Natl. Acad. Sci. USA* **93,** 10240–10245.

Witty, K., Wright, J. H., and Matrisian, L. M. (1995). Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell* **6,** 1287–1303.

Wu, T., Vasvada, R., Yang, K., Massfelder, T., Ganz, M., Abbas, S., Care, A., and Stewart, A. (1996). Structural and physiologic characterization of the mid-region secretory form of PTHrP. *J. Biol. Chem.* **271,** 24371–24381.

Wu, T. L., Insogna, K. L., Hough, L. M., Milstone, L., and Stewart, A. F. (1987). Skin-derived fibroblasts respond to human parathyroid hormone-like adenylate cyclase-stimulating proteins. *J. Clin. Endocrinol. Metab.* **65,** 105–109.

Wysolmerski, J. J., and Broadus, A. E. (1994). Hypercalcemia of malignancy: The central role of parathyroid hormone related protein. *Annu. Rev. Med.* **45,** 189–200.

Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E., and Philbrick, W. M. (1996). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* **121,** 3539–3547.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., Karaplis, A., and Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Development* **125,** 1285–1294.

Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. (1995). Sequential requirement for hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131,** 215–226.

Received for publication February 23, 1998
Revised June 26, 1998
Accepted July 22, 1998

Development 126, 3485-3493 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV4185

Parathyroid hormone-related protein signaling is necessary for sexual dimorphism during embryonic mammary development

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Accepted 2 June; published on WWW 19 July 1999

SUMMARY

Male mice lack mammary glands due to the interaction of circulating androgens with local epithelial-mesenchymal signaling in the developing mammary bud. Mammary epithelial cells induce androgen receptor (AR) within the mammary mesenchyme and, in response to androgens, the mesenchyme condenses around the epithelial bud, destroying it. We show that this process involves apoptosis and that, in the absence of parathyroid hormone-related protein (PTHrP) or its receptor, the PTH/PTHrP receptor (PPR1), it fails due to a lack of mesenchymal AR expression. In addition, the expression of tenascin C, another marker of the mammary mesenchyme, is also dependent on PTHrP. PTHrP expression is initiated on E11 and, within the ventral epidermis, is restricted to the forming mammary epithelial bud. In contrast, PPR1 expression is not limited to the mammary bud, but is found generally within the subepidermal mesenchyme. Finally, transgenic overexpression of PTHrP within the basal epidermis induces AR and tenasin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP can induce the ventral mesenchyme to express mammary mesenchyme markers. We propose that PTHrP expression specifically within the developing epithelial bud acts as a dominant signal participating in cell fate decisions leading to a specialized mammary mesenchyme.

Key words: Androgen receptor, Tenascin C, Epithelial-mesenchymal interaction, Apoptosis, PTH/PTHrP receptor, Mouse

INTRODUCTION

The development of many epithelial organs depends on a series of sequential and reciprocal interactions between epithelial cells and adjacent mesenchymal or stromal cells (Thesleff et al., 1995; Birchmeier and Birchmeier, 1993). The mammary gland is an example of an organ where these epithelial-mesenchymal interactions play a critical role, especially during embryonic development (Sakakura, 1987; Cunha, 1994; Robinson et al., 1999). In mice, mammary development commences with the formation of 5 pairs of epithelial buds located on the ventral surface of the embryo. Each bud begins as a localized thickening of the epidermis first noted on embryonic day 10 (E10, appearance of the vaginal plug = E0), and between E10 and E12-13 this initial placode invaginates into the underlying mesenchyme and the mammary epithelial cells organize themselves into a characteristic "light-bulb" shape (Sakakura, 1987; Robinson et al., 1999). Initially, the mammary mesenchyme is indistinguishable from the ventral dermal mesenchyme, but by the time the mammary epithelial bud is fully formed, it is invested by several layers of mesenchymal cells that are morphologically and functionally distinct from the surrounding dermal mesenchyme (Sakakura, 1987). Recombination experiments have documented that the mammary epithelium and mesenchyme contribute to the formation of each other during mammary bud development and, within the mature mammary bud (through E14-15), each compartment retains the capacity to induce fully the formation of the other (Propper and Gomot, 1967; Heuberger et al., 1982; Cunha et al., 1995).

One of the best-studied aspects of epithelial-mesenchymal interaction during murine mammary development is the androgen-mediated destruction of the mammary bud in males. In male embryos, beginning on E14, the mammary mesenchyme condenses around the neck of the epithelial bud and disrupts the stalk connecting the mammary bud to the overlying epidermis (Turner and Gomez, 1933; Sakakura, 1987). In most strains of mice, the mammary epithelial remnant subsequently degenerates and no nipple is formed, explaining the lack of nipples and mammary glands in adult males (Sakakura, 1987). However, the degree to which the epithelial remnant is destroyed is variable and, in rats, while the stalk is destroyed, there is little degeneration of the remaining epithelium. Several studies have shown that this process occurs as a result of the secretion of androgens by the fetal testes, which act directly on the mammary mesenchyme to trigger its condensation (Raynaud and Frilley, 1947; Raynaud, 1949; Hoshino, 1965; Neuman et al., 1970;

Kratochwil, 1977; Kratochwil and Schwartz, 1977). It has also been demonstrated that the epithelium is a necessary participant in this process, which instructs the mesenchyme to express androgen receptors (Heuberger et al., 1982). Although it is clear that the destruction of the mammary buds by androgens is dependent on a bi-directional flow of information between epithelium and mesenchyme, the identity of the signals exchanged is not known.

One molecule that has recently been implicated in epithelial-mesenchymal interactions at several sites during development is parathyroid hormone-related protein (PTHrP; Wysolmerski and Stewart, 1998). PTHrP was originally discovered as the cause of hypercalcemia in patients with a variety of cancers and it derives its name from a common ancestry shared with parathyroid hormone (PTH; Philbrick et al., 1996). PTHrP also shares the same family of G-protein-coupled receptors with PTH. The prototype of this family is the Type I PTH/PTHrP receptor (PPR1), which appears to subserve the majority of the known functions of PTHrP (Jüppner et al., 1991: Philbrick et al., 1996). During development, PTHrP has been shown to be produced by many developing epithelial structures, while the PPR1 is expressed on adjacent mesenchymal cells, suggesting a role for PTHrP in epithelial-to-mesenchymal signaling, a notion recently confirmed by several PTHrP transgenic and knockout mouse models (Lee et al., 1995; Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994, 1995, 1998).

We have recently shown that PTHrP is necessary for mammary gland development. In the absence of PTHrP or its receptor, there is a failure of the initiation of ductal branching morphogenesis and nipple formation during embryonic mammary development (Wysolmerski et al., 1998). In PTHrP or PPR-1 knockout embryos the mammary bud initially forms normally, but it fails to undergo the primary growth spurt, and the mammary epithelial cells degenerate and disappear before birth (Wysolmerski et al., 1998). Overexpression of PTHrP within the mammary gland also affects branching morphogenesis, leading to an impairment of hormonally stimulated ductal proliferation and side-branching during puberty and early pregnancy (Wysolmerski et al., 1995). Both during embryonic development and during puberty, PTHrP is produced by epithelial cells, while the PPR1 resides on mesenchymal cells during embryonic development and fat pad and periductal stromal cells during puberty (Wysolmerski et al., 1998; Dunbar et al., 1998). Given the patterns of expression of PTHrP and the PPR1 during the early stages of mammary development, and given the requirement for epithelial-mesenchymal interaction in the androgen-mediated destruction of the mammary bud, we initiated a study of PTHrP's possible involvement in this process.

In this report, we document that PTHrP and the PPR1 are necessary for the normal sexual dimorphism seen during murine mammary development. PTHrP is expressed specifically within the epithelial cells of the mammary bud concurrent with its formation, and we identify it to be an epithelial signal responsible for inducing androgen receptor and tenascin C expression within the mammary mesenchyme. Ectopic expression of PTHrP within the fetal epidermis results in the expression of mammary mesenchyme markers in the fetal dermis. These findings suggest that PTHrP participates in regulating the mesenchymal cell fate decisions that result in the formation of a specialized mammary mesenchyme.

MATERIALS AND METHODS

Mouse strains

The disrupted PTHrP and PPR1 alleles were progressively bred onto a CD-1 background to improve litter size and embryo survival, and mice heterozygous for these alleles were mated to produce homozygous PTHrP- and PPR1-null embryos (appearance of vaginal plug=day 0). Wild-type littermates were used as normal controls. Embryos were removed from the uterus and genotyped using the polymerase chain reaction as described previously (Wysolmerski et al., 1998). Keratin 14 (K14) is expressed in specific subsets of epithelial cells, including fetal mammary epithelial cells and basal keratinocytes of the skin, and we have previously documented that the K14 promoter can successfully target PTHrP transgene expression to these cells (Wysolmerski et al., 1998). In the present study, K14-PTHrP embryos were identified as reported previously, and K14-PTHrP/PTHrP-null embryos were produced by mating mice both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null allele with mice heterozygous for the disrupted PTHrP allele (Wysolmerski et al., 1998). All embryos were sexed both by visual inspection of the gonads and by amplification of a 240 bp fragment of the SRY gene by PCR (Wysolmerski et al., 1998).

Histology/immunohistochemistry

After harvesting, embryos were fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was removed and the intact mammary glands were dissected and embedded in paraffin. Appropriate sections were identified by serial sectioning and hematoxylin and eosin staining, and immunohistochemistry was performed using standard techniques. The androgen receptor antibody is a rabbit polyclonal and was the kind gift of Dr Gail Prins (The University of Illinois at Chicago, Chicago, Illinois). Primary incubations with the androgen receptor antibody were performed at 4°C for 12 hours at a concentration of 0.5 or 1.0 μg/ml and were preceded by boiling of the sections for 30 minutes in 0.01 M citrate buffer pH 6.0. Competition experiments were performed with AR21, which consists of the first 21 amino acids of the androgen receptor and contains the antibody epitopes, and with peptide AR462, which consists of amino acids 462-478 and does not contain the epitopes (peptides courtesy of Dr Prins). The tenascin C antibody is also a rabbit polyclonal antiserum and was the kind gift of Drs Toshimichi Yoshida and Teruyo Sakakura (Mie University, Tsu, Japan). Primary incubations were performed at a concentration of 2.5 or 5.0 μg/ml at room temperature for 1 hour and were preceded by a 10 minute incubation in 0.1% trypsin in 0.1% (w/v) calcium chloride pH 7.8. Primary antibodies were detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and either 3, 3′ diaminobenzidine or TrueBlue™ peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as chromagens. Apoptosis was detected by terminal deoxytransferase labeling (TUNEL assay) utilizing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Indianapolis, IN).

In situ hybridization

In situ hybridization on paraffin sections was performed as previously described (Dunbar et al., 1998; Wysolmerski et al., 1998). Probes were generated from a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the type I PTH/PTHrP receptor gene (Dunbar et al., 1998; Wysolmerski et al., 1998). Whole-mount in situ hybridization was performed using a protocol kindly provided by Dr Trevor Dale (Phippard et al., 1996). In brief, embryos were harvested, fixed for 2 hours in 4% paraformaldehyde at room temperature, treated with proteinase K (20 μg/ml) for 10-15 minutes

at room temperature and postfixed in 4% paraformaldehyde/0.1% glutaraldehyde for 20 minutes at room temperature. The embryos were then hybridized with digoxigenin-labeled riboprobes for PTHrP and PPR1 generated from the templates described above using the Genius kit (Boehringer Mannheim, Indianapolis, IN). The hybridization buffer consisted of 50% formamide, 1.3× SSC, 5 mM EDTA, 0.2% Tween 20, 0.5% CHAPS and 50 μg/ml yeast RNA and the hybridization was at 70°C overnight. Samples were then washed twice in hybridization buffer for 30 minutes at 70°C, once in 1:1 hybridization buffer:TBST at 70°C for 20 minutes and twice in TBST at room temperature for 30 minutes. Following these washes, the embryos were incubated in blocking solution consisting of 10% sheep serum and 1 mg/ml BSA in TBST for 3 hours at room temperature and then were incubated with anti-digoxigenin antiserum (Genius kit, Boehringer Mannheim, Indianapolis, IN) overnight at 4°C. The color reaction was performed according to the manufacturer's protocol and signals developed between 1 and 2 hours.

RESULTS

PTHrP and the PTH/PTHrP receptor are necessary for sexual dimorphism during murine mammary development

In order to ascertain if PTHrP signaling contributed to the androgen-mediated destruction of the male mammary bud, we examined the gross appearance of the mammary buds in PTHrP- and PPR 1-knockout mice. We performed this analysis on male embryos at E15, a time point at which the destruction of the mammary buds should normally be well advanced (Sakakura, 1987). First, we examined 48 male embryos resulting from crosses between heterozygous PTHrP-null parents. In all 10 wild-type embryos, the mammary buds were either completely absent or consisted of very small remnants. In stark contrast, in each homozygous PTHrP-knockout embryo, all mammary buds were present, well preserved and indistinguishable from those observed in female embryos. There was little evidence of haplotype insufficiency, since only one of 28 heterozygous PTHrP-knockout embryos failed to demonstrate the expected destruction of the mammary buds. We next examined 10 male PPR 1-knockout embryos and found that they uniformly also had the abnormal persistence of mammary buds at E15.

The histological findings in these embryos are shown in Fig. 1. Fig. 1A shows the typical appearance of a wild-type female mammary bud at E15. In contrast, at E15, the wild-type male bud is actively being destroyed (Fig. 1B). There is extensive mesenchymal condensation above the epithelial remnant in the region where the bud stalk appears to be degenerating. The stalk has been interrupted and the epithelial remnant, which is misshapen and degenerating (see TUNEL data below), is no longer connected to the epidermis. However, in PTHrP- and PPR 1-knockout males (Fig. 1C,D), the mammary buds appear similar to those seen in female embryos. In these embryos, there is no mesenchymal cell condensation, and the mammary mesenchyme continues to consist of several layers of cells arrayed concentrically around the epithelial bud. In addition, the epithelial stalk is intact, and the mammary epithelial cells maintain their connection with the epidermis. The mutant male buds persist until E16-E17, at which point they fail to undergo the initial round of branching morphogenesis and instead degenerate, findings identical to that previously described for female PTHrP and PPR-1 knockout mammary rudiments (data not shown) (Wysolmerski et al., 1998).

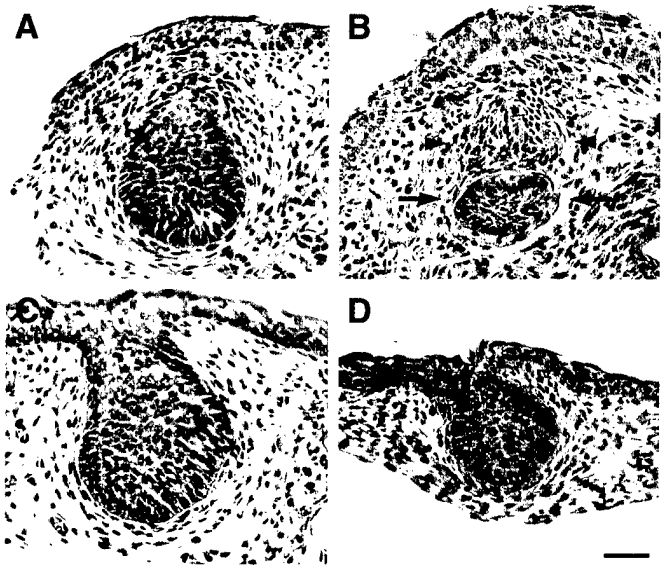

Fig. 1. Failure of the androgen-mediated destruction of the mammary buds in PTHrP- and PPR1-knockout embryos. Hematoxylin- and eosin-stained sections through E15 mammary buds taken from a wild-type female embryo (A), a wild-type male embryo (B), a PTHrP-knockout male embryo (C) and a PPR1-knockout male embryo (D). In the normal male embryo (B), the mesenchymal cells have condensed around the degenerating epithelial stalk (between arrowheads) and the epithelial remnant is misshapen and disconnected from the epidermis (arrows). In contrast, note the lack of mesenchymal condensation and the well preserved epithelial stalks in C and D. Scale bar, 110 μm.

The destruction of the mammary bud in male embryos is an example of programmed cell death (Fig. 2). In the wild-type male bud at E15 (Fig. 2A), there is widespread TUNEL staining in the region of the degenerating epithelial stalk. This appears to involve both the epithelial cells of the stalk and the mesenchymal cells within the androgen-induced condensation. In addition, there is evidence of apoptosis occurring within the epithelial remnant that lies beneath the epidermis. In contrast, in PTHrP-knockout males (Fig. 2B), there is no apoptosis. Similar results were obtained in PPR-1 knockout embryos and, in both strains of knockout mice, the lack of TUNEL-staining was identical to the results obtained with wild-type female embryos (results not shown). Therefore, in the absence of PTHrP or the Type 1 PTH/PTHrP receptor, the pattern of sexual dimorphism normally observed during early mammary development is abolished.

PTHrP and PPR1 are necessary for androgen receptor and tenascin C expression in the dense mammary mesenchyme

The androgen-mediated destruction of the mammary bud is dependent on the presence of functional androgen receptors within the dense mammary mesenchyme, and the expression of these receptors is induced by signals from the mammary epithelium (Heuberger et al., 1982; Sakakura, 1987). The absence of an androgen response in the PTHrP- and PPR1-knockout buds combined with the epithelial expression of

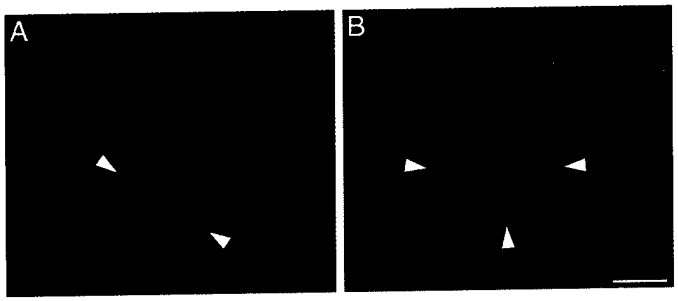

Fig. 2. Programmed cell death in male mammary buds at E15. (A) Results of a TUNEL assay performed on a section through a wild-type male mammary bud. Apoptotic nuclei stain bright green whereas normal nuclei stain pale green. Note the multitude of apoptotic nuclei in the region of the stalk and mesenchymal condensation. Note that there is apoptosis also occurring in the epithelial remnant (between arrowheads). (B) Results of a TUNEL assay performed on a section through a PTHrP-knockout male mammary bud. Note that the mammary bud (outlined by arrowheads) is well preserved and that there is no apoptosis, as demonstrated by the lack of bright green nuclei, in either the epithelial bud or in the mammary mesenchyme. Scale bar, 80 μm.

PTHrP and the mesenchymal expression of the PPR1 (Dunbar et al., 1998; Wysolmerski et al., 1998), led us to posit that PTHrP might be the epithelial signal responsible for the induction of androgen receptor expression within the dense mammary mesenchyme. To investigate this possibility, we examined androgen receptor expression in the mammary buds of wild-type female embryos and male and female PTHrP- and PPR1-knockout embryos at E15 by immunohistochemistry (Fig. 3A-C). In the wild-type bud (Fig. 3B), one can appreciate the intense nuclear staining for androgen receptor in the cells comprising the dense mammary mesenchyme. There is no staining in the general dermal mesenchyme. This pattern of androgen receptor localization is identical to that seen in previous studies using [^{3}H]testosterone autoradiography (Heuberger et al., 1982). In contrast, this staining pattern is absent in the PTHrP- (Fig. 3A) or PPR1-knockout (Fig. 3C) buds. In these glands, there are only occasional nuclei that stain weakly for androgen receptor within the mesenchymal cells closest to the epithelial basement membrane. The absence of androgen receptor staining appears to be specific for the mammary mesenchyme, for androgen receptor staining is normal within the testes of Col II-PTHrP/PTHrP-null (Col II-rescued) mice that lack PTHrP in all tissues except the skeleton (data not shown) (Majdic et al., 1995; Philbrick et al., 1998; Wysolmerski et al., 1998). Furthermore, the development of the Wolffian ducts and the descent of the fetal testes are normal in the absence of either PTHrP or the PPR1, demonstrating an intact androgen response in these tissues (Gilbert, 1994; Grumbach and Conte, 1992).

Androgen receptor expression is one of the characteristics of the mammary mesenchyme that sets it apart from the dermal mesenchyme (Sakakura, 1987), so that the absence of androgen receptor expression within the mammary mesenchyme of PTHrP- and PPR1-knockout embryos suggested that there might be more fundamental defects in the differentiation of these cells. The other classic marker of the specialized mammary mesenchyme is tenascin C (Sakakura, 1987). Therefore, we next examined PTHrP- and PPR1-knockout mammary buds for the expression of this extracellular matrix protein by immunohistochemistry (Fig. 3D-F). The results were similar to those seen with respect to androgen receptor expression. The wild-type epithelial bud (Fig. 3E) was surrounded by a halo of tenascin C within the extracellular matrix of the dense mammary mesenchyme, but not within the general dermal matrix. In contrast, there was no tenascin C expression surrounding the PTHrP- (Fig. 3D) and PPR1- knockout buds (Fig. 3F), suggesting that, in the absence of PTHrP or PPR1, the dense mammary mesenchyme does not differentiate properly. As with androgen receptor expression, there did not appear to be a generalized defect in tenascin C expression, as there was ample tenascin staining in the developing bones of knockout embryos (data not shown) (Erickson and Bourdon, 1989).

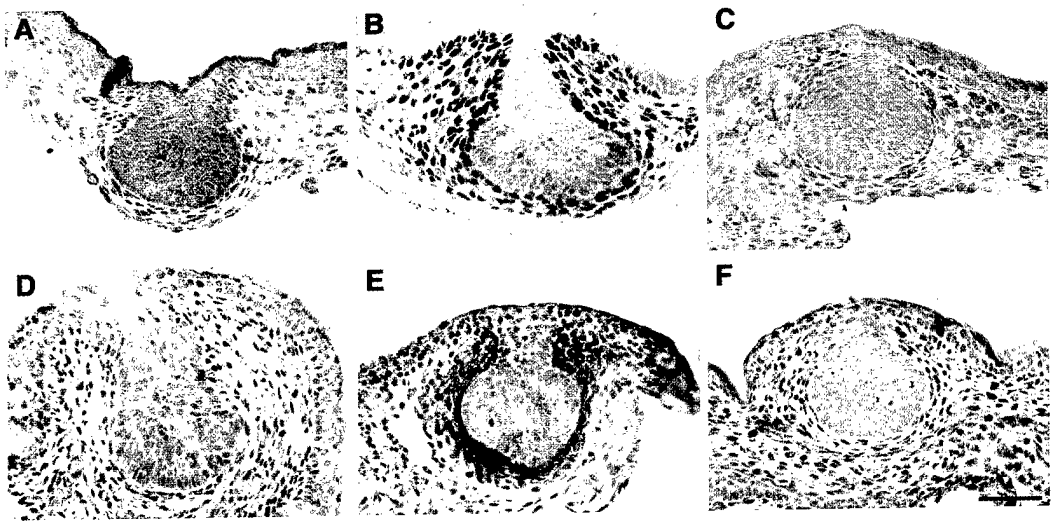

Fig. 3. Androgen receptor and tenascin C staining of E15 mammary buds. (A-C) Sections stained for androgen receptor and (D-F) represent sections stained for tenascin C. (A,D) Sections through male PTHrP-knockout mammary buds. (B,E) Sections through female wild-type buds. (C,F) Sections through male PPR1-knockout mammary buds. Again, note the well-preserved mammary buds in the male knockouts. There is prominent nuclear staining for androgen receptor in the dense mammary mesenchyme of the wild-type bud in B, but little or no androgen receptor staining in the PTHrP-knockout bud in A and the PPR-1-knockout bud in C. Likewise, there is prominent staining for tenascin C in the extracellular matrix surrounding the wild-type bud (E), but an absence of similar staining in the knockouts (D,F). Scale bar, 120 μm.

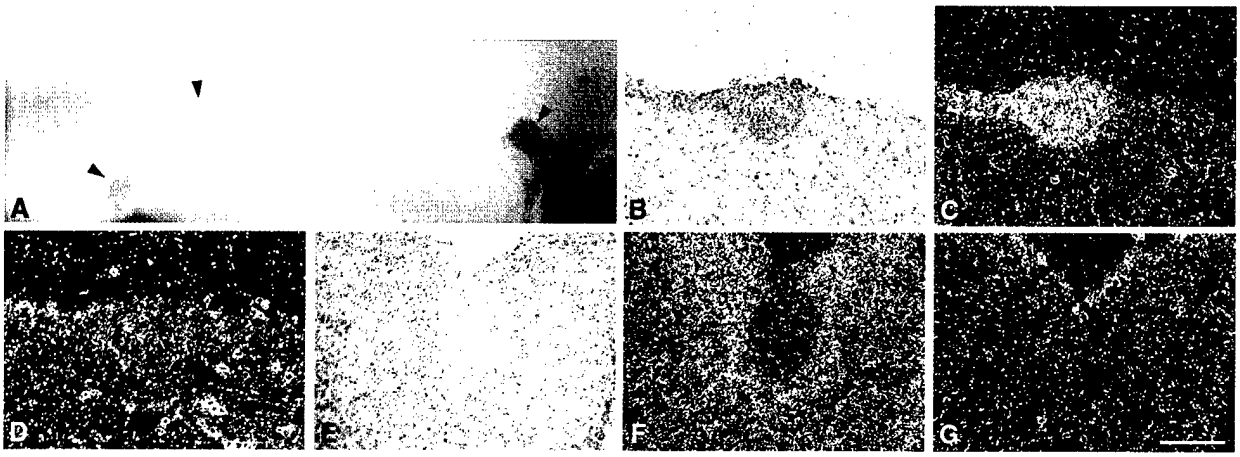

Fig. 4. Initiation of PTHrP expression in developing mammary buds. (A) A representative photograph of whole-mount in situ hybridization experiments using PTHrP antisense probe on a late E11 embryo. The photograph shows a magnified view of the ventral-lateral surface of the embryo. PTHrP expression is not seen within the general epidermis, but is limited to the mammary buds (arrowheads). (B-D) In situ hybridization for PTHrP on sections through mammary buds taken from E12 embryos. (B,C) Bright-field and dark-field views of the same section, which was hybridized to a PTHrP antisense probes. (D) Section hybridized to a PTHrP sense probe. Note that PTHrP is expressed within the epithelial cells of the mammary bud. (E-G) In situ hybridization for the PPR1 on sections through mammary buds taken from E12 embryos. (E,F) Bright-field and dark-field views of the same section, which was hybridized to a PPR1 antisense probes. (G) Section hybridized to a PPR1 sense probe. Note that the PPR1 is expressed throughout the ventral dermal mesenchyme as well as within the dense mammary mesenchyme. Scale bars, (A) 380 μm; (B-G) 150 μm.

PTHrP is expressed specifically within the forming mammary epithelium

We reasoned that, if PTHrP were to participate in regulating the differentiation of the dense mammary mesenchyme, it should be expressed early during the formation of the mammary bud. In mice, this process is initiated on E10 and is complete by E14-15. We have previously demonstrated that PTHrP is expressed within the mammary epithelium in the fully formed mammary bud (Dunbar et al., 1998; Wysolmerski et al., 1998). To detect the onset of PTHrP expression during the formation of the mammary bud, we performed whole-mount in situ hybridization on wild-type embryos from E10-E12. There was no expression of PTHrP in the ventral epidermis until late on E11, after the mammary buds had already begun to form and, by late E11-E12, there was strong and specific hybridization for PTHrP within the developing mammary buds (Fig. 4A). In situ hybridization on sections through developing mammary buds confirmed these findings, demonstrating that PTHrP was expressed in the mammary epithelial cells invaginating into the underlying mesenchyme (Fig. 4B-D). There was little, if any, expression of PTHrP within the ventral epidermis apart from the mammary buds at these stages. These findings are identical to those obtained by other investigators in whole-mount in situ experiments performed on E13 embryos (K. Lee and G. Segre, personal communication). PPR1 expression was found throughout the ventral mesenchyme both underlying the epidermis and surrounding the mammary buds (Fig. 4E-G).

Re-expression of PTHrP re-establishes sexual dimorphism

PTHrP and PPR1 are both expressed within the embryo as early as the morula stage. Therefore, it is possible that the changes that we observed in the knockout embryos were not

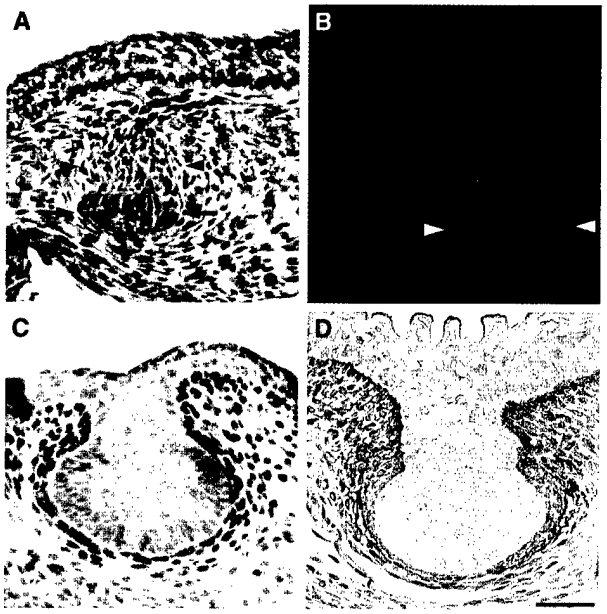

Fig. 5. Return of sexual dimorphism in K14-PTHrP/PTHrP-null embryos. (A) Histology of a mammary bud taken from a male K14-PTHrP/PTHrP-null embryo at E15. Note the typical mesenchymal condensation around the epithelial stalk (between arrowheads) and the degenerating epithelial remnant (between arrows) (compare with Fig. 1B). (B) TUNEL assay performed on a male K14-PTHrP/PTHrP-null embryo at E15. Note the return of the apoptotic response in both the mammary mesenchyme and the epithelial remnant (arrowheads). (C) Androgen receptor staining in a female K14-PTHrP/PTHrP-null embryo at E15. (D) Tenascin C staining in a female K14-PTHrP/PTHrP-null embryo at E15. Note that with the restoration of PTHrP to the mammary epithelium, both molecules are again induced within the mammary mesenchyme. Scale bar,100 μm.

the result of the loss of PTHrP-signaling from mammary epithelium to mammary mesenchyme during the formation of the mammary bud, but were instead the consequence of earlier changes in mesenchymal patterning (de Stolpe et al., 1993; Behrendtsen et al., 1995). In order to demonstrate a direct link between PTHrP production by the mammary epithelium and mesenchymal cell differentiation, we utilized transgenic mice overexpressing PTHrP under the control of the keratin 14 promoter (K14-PTHrP mice) to restore PTHrP to the mammary epithelium of PTHrP-knockout embryos (Wysolmerski et al., 1998). We have previously shown that this promoter directs transgene expression to the epithelial cells of the fetal mammary gland and have recently observed that a K14-driven β-galactosidase transgene is expressed within the mammary bud as early as E12 (P. R. D. and J. J. W., unpublished results). Therefore, we reasoned that a K14-transgene replacement strategy would be expected to duplicate the normal expression of PTHrP within the mammary bud. The K14-PTHrP transgene was bred onto a homozygous PTHrP-null background, producing embryos (K14-PTHrP/PTHrP-null) that were devoid of PTHrP in all tissues except for the sites of K14 expression (such as mammary epithelial cells). As depicted in Fig. 5, the reintroduction of PTHrP expression within the mammary epithelium resulted in a return of the androgen-mediated destruction of the mammary buds and re-established androgen and tenascin C expression within the dense mammary mesenchyme. On a gross level, at E15, male K14-PTHrP/PTHrP-null embryos possessed only remnants of mammary buds. Histologically, these buds demonstrated the typical features of the androgen-mediated response (Fig. 5A), and TUNEL staining revealed a return of the apoptotic response (Fig. 5B). Androgen receptor (Fig. 5C) and tenascin C (Fig. 5D) staining of female K14-PTHrP/PTHrP-null mammary buds at E15 showed the expected pattern of expression of these markers in the mammary mesenchyme (compare Fig. 5C,D with Fig. 3A,D). These results demonstrate that it is the expression of PTHrP within the epithelium during mammary bud formation that is critical for the normal pattern of sexual dimorphism and suggest that PTHrP signaling from the epithelium to the mesenchyme during early mammary gland development is required for full differentiation of the mammary mesenchyme.

Ectopic expression of PTHrP induces ectopic expression of mammary mesenchyme markers

The mammary phenotypes of the PTHrP- and PPR1-knockout embryos, the specific expression of PTHrP within the mammary epithelial buds and the general expression of the PPR1 within the subepidermal mesenchyme suggested that PTHrP might serve as a dominant signal regulating the fate or course of differentiation of the ventral mesenchyme. We hypothesized that the presence of PTHrP in the mammary bud might lead to the acquisition of the mammary mesenchyme phenotype, while the absence of PTHrP within the general epidermis would be associated with a dermal mesenchyme phenotype. If this were true, ectopic expression of PTHrP within the epidermis might lead to the formation of mammary mesenchyme instead of dermis. In order to test this idea, we again turned to K14-PTHrP transgenic mice. As demonstrated in Fig. 6A, K14 is expressed not only within the mammary epithelium but also within the basal keratinocytes of the fetal epidermis (Kopan and Fuchs, 1989). Therefore, we stained the epidermis of K14-PTHrP transgenic and wild-type littermates for the expression of androgen receptor and tenascin C. In wild-type embryos, we could not detect androgen receptor expression within the ventral dermal mesenchyme but, in the ventral epidermis of K14-PTHrP transgenics, there was clear expression of androgen receptor within the nuclei of the dermal mesenchymal cells closest to the epidermis (Fig. 6B,C). As has been previously reported, there was some tenascin C expression within the basement membrane of the ventral epidermis in wild-type embryos, especially around developing hair follicles (Fig. 6E). However, there was a dramatic accumulation of tenascin C within the basement membrane of the ventral epidermis in K14-PTHrP transgenic embryos as well as an accumulation of tenascin C within the extracellular matrix of the dermal mesenchyme just beneath the epidermis (Fig. 6F). Despite the widespread expression of the PPR1 beneath the epidermis (Fig. 4F,G), only those cells nearest the epidermal source of PTHrP expressed androgen receptor (Fig. 6C) or tenascin C (Fig. 6F), demonstrating that PTHrP acted only over a range of a few cell diameters. Interestingly, these effects also appeared to be limited to the ventral epidermis of the K14-PTHrP embryos. Staining of the dorsal epidermis did not reveal expression of androgen receptor or tenascin C within the dermal mesenchyme (Fig. 6D,G). This was surprising, since both the PPR1 gene and the K14-PTHrP transgene were expressed in both dorsal and ventral epidermis (data not shown). Thus, ectopic overexpression of PTHrP within the ventral epidermis leads to the ectopic expression of androgen receptor and tenascin C within mesenchymal cells that should posses a dermal phenotype, suggesting that, at least on the ventral surface of the embryo, PTHrP may act as a dominant signal to induce the differentiation of the mammary mesenchyme.

DISCUSSION

In this report, we demonstrate that PTHrP, signaling through the PPR1, is essential for the sexual dimorphism in normal murine mammary development. In PTHrP- or the PPR1-knockout embryos, the androgen-mediated destruction of the mammary bud in male embryos fails, due to the absence of androgen receptors in the mammary mesenchyme. In addition, the mammary buds of both types of knockout mice lack tenascin C, an extracellular matrix constituent which is highly expressed within the mammary mesenchyme but not within the dermis (Sakakura, 1987). PTHrP is expressed within the epithelial cells of the forming mammary bud beginning late on E11, however it is not expressed within the mesenchyme or within the epidermis at this stage. In contrast to PTHrP, the PPR1 is expressed within the mesenchyme and its expression is not restricted to the developing mammary bud. Rather, it is expressed both within the mesenchyme surrounding the mammary bud and within the mesenchyme underlying the epidermis. The expression of androgen receptor and tenascin C are directly dependent on PTHrP expression during the formation of the mammary bud and are not a consequence of earlier PTHrP expression, for transgenic expression of PTHrP within the forming mammary epithelial bud in otherwise PTHrP-null (K14-PTHrP/PTHrP-null) embryos leads to the

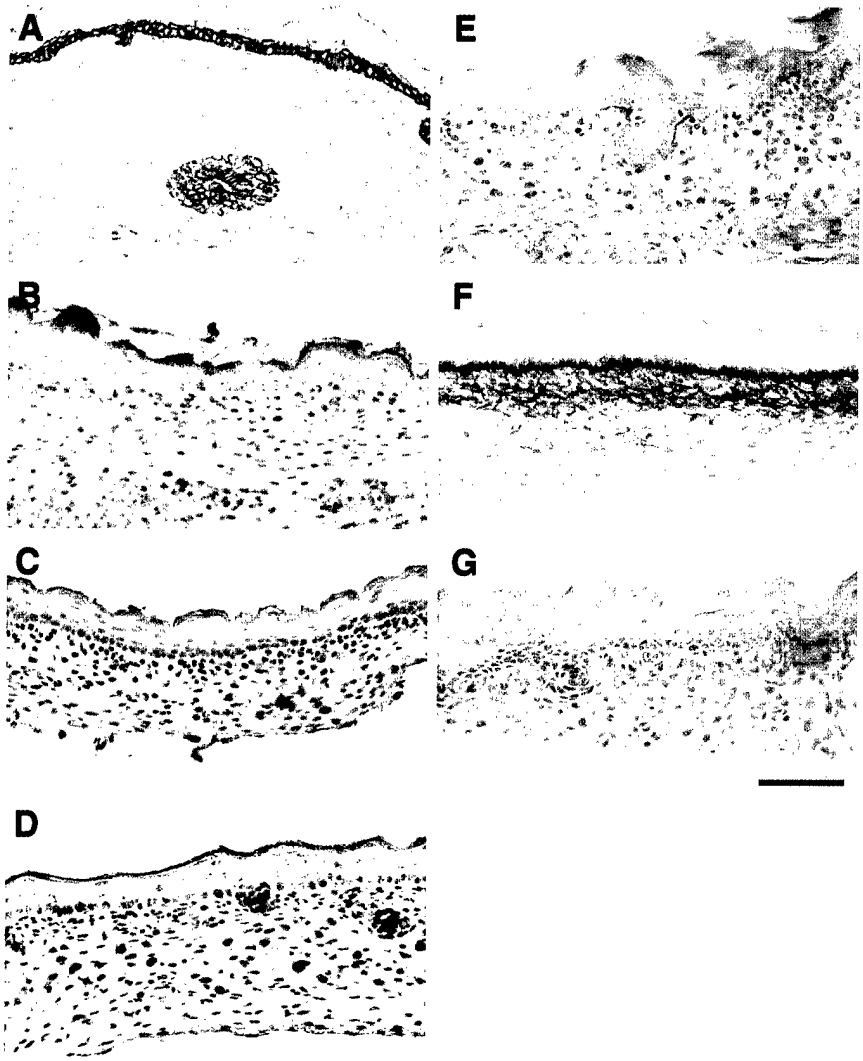

Fig. 6. Ectopic expression of PTHrP in the epidermis induces dermal expression of androgen receptor and tenascin C. (A) Immunohistochemistry for keratin 14 in the fetal epidermis at E18. Note that K14 is expressed both within the fetal mammary epithelial cells within the mammary duct below the epidermis and within the basal keratinocytes of the epidermis. (B-D) Androgen receptor staining of ventral epidermis from a wild-type embryo at E18 (B), and of ventral (C) and dorsal (D) epidermis from a K14-PTHrP transgenic embryo, also at E18. There is no androgen receptor staining in the wild-type dermis (B), but there is nuclear androgen receptor staining in the dermal cells close to the epidermal basement membrane in the ventral surface of the K14-PTHrP transgenic. However, this is not true for the dorsal aspect of the K14-PTHrP embryos as seen in D. (E-G) A similar pattern is seen for tenascin C. In the ventral surface of wild-type embryos at E18 (E), there is some tenascin expression along the basement membrane, especially in the vicinity of developing hair follicles. However, there is a dramatic upregulation of tenascin within the basement membrane and within the extracellular matrix of the upper dermis on the ventral surface of K14-PTHrP embryos at E18 (F), but not on the dorsal surface of K14-PTHrP transgenic embryos (G). Scale bar, 150 μm.

restoration of the expression of both androgen receptor and tenascin C, and thus the androgen-mediated destruction of the mammary bud. Finally, transgenic expression of PTHrP in the basal epidermis leads to the induction of androgen receptor and tenascin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP may induce an ectopic mammary mesenchyme phenotype.

The expression of androgen receptors and tenascin C has classically distinguished the dense mammary mesenchyme from the surrounding dermal mesenchyme (Sakakura, 1987). It has been known for many years that the mesenchymal expression of both molecules was dependent on short-range inductive tissue interactions with the mammary epithelium, but the nature of the inductive signal(s) sent from epithelium to mesenchyme was not known (Heuberger et al., 1982; Inaguma et al., 1988; Kalembey et al., 1997). Our findings suggest that PTHrP is a vital component in these interactions. However, both of these molecules are expressed elsewhere and their expression is not universally dependent on PTHrP. Likewise, the ability of epidermal overexpression of PTHrP to induce the production of these molecules does not appear to extend to all the dermal mesenchyme, for we did not observe their induction within the dorsal subcutis. Therefore, it is unlikely that PTHrP generally regulates the expression of these molecules. Rather, our hypothesis is that PTHrP, expressed exclusively within the developing epithelial bud, acts as a short-range dominant signal to a receptive ventral mesenchyme to differentiate into dense mammary mesenchyme. This results in the induction of mammary mesenchyme-specific genes (e.g. tenascin and androgen receptor) and the ability of the mesenchyme to support mammary epithelial morphogenesis.

In addition to the failure of androgen responsiveness, the loss of PTHrP-signaling also renders the mammary mesenchyme incapable of supporting the initiation of branching morphogenesis associated with the primary growth spurt on E16 (Dunbar et al., 1998; Wysolmerski et al., 1998). It is unlikely that the loss of either androgen receptor or tenascin C expression explains the inability of the mammary mesenchyme to support the outgrowth of the mammary epithelium in female PTHrP- or PPR1 knockouts because Tfm mice with inactivating mutations of the androgen receptor as well as tenascin C-knockouts both carry out these processes normally (Kratochwil and Schwartz, 1977; Saga et al., 1992). Recently, a series of additional molecules such as BMP-4,

preprotachykinin, Msx 2, Fgf 7, Hoxa9, Hoxb9 and Hoxd9 have been described to be expressed in the mammary mesenchyme (Phippard et al., 1996; Weil et al., 1995; Cunha and Hom, 1996; Robinson et al., 1999; Chen and Capecchi, 1999). However, there is no evidence to date to suggest that the deletion of any of these molecules phenocopies the changes in mammary development noted in the PTHrP- and PPR1-knockouts (Robinson et al., 1999; Chen and Cappechi, 1999). The mammary phenotype of LEF-1-deficient mice remains the closest to that of the PTHrP and PPR1 knockouts (van Genderen et al., 1994; Kratochwil et al., 1996). However, LEF-1 is expressed in the mammary epithelium prior to the onset of PTHrP expression and the failure of mammary development in LEF-1-knockout embryos occurs at an earlier stage than does the failure of mammary development in PTHrP- or PPR1-knockouts. Thus, if LEF-1 and PTHrP are in a common genetic pathway, LEF-1 most likely resides upstream of PTHrP within this pathway (van Genderen et al., 1994; Kratochwil et al., 1996). Except for the expression of androgen receptors and the androgen-mediated destruction of the mammary bud, the nature of the other PTHrP-induced mesenchymal changes that allow the mammary mesenchyme to support morphogenesis remains obscure.

It is also apparent from our results that, although the mammary mesenchyme is dependent on PTHrP for its ability to support morphogenesis, the morphological appearance of the mammary mesenchyme is not dependent on PTHrP. In both PTHrP- and PPR1-knockout embryos, the mammary mesenchyme is histologically indistinguishable from that in normal littermates. The most-likely explanation for these findings is that the condensation or "structural" differentiation of the mammary mesenchyme precedes its functional differentiation (which is dependent on PTHrP). In support of this concept, we have recently observed that syndecan 1, which has been reported to be important to the condensation of tooth mesenchyme, continues to be expressed normally within the mammary mesenchyme of PTHrP and PPR1 knockouts (P. R. D., unpublished observations; Salmivirta et al., 1991; Thesleff et al., 1995). It is likely that there are one or more reciprocal exchanges between the mammary epithelium and the mammary mesenchyme that precede the actions of PTHrP and it will be important to examine the mechanisms leading to activation of PTHrP expression within the developing epithelial bud.

In summary, we have found that PTHrP and the PPR1 are necessary for sexual dimorphism during murine mammary development. PTHrP is an inductive signal from the epithelium to the mesenchyme that is necessary for androgen receptor and tenascin C expression within the mesenchyme. We propose that PTHrP participates in the regulation of mesenchymal cell fate decisions leading to a distinct mammary mesenchyme with the ability to support early mammary morphogenesis. The specific initiation of PTHrP gene expression within the epithelium of the forming mammary bud, the more general expression of the PPR1 within the subepidermal mesenchyme, the ability of ectopic epidermal expression of PTHrP to induce the inappropriate dermal expression of androgen receptor and tenascin C, the short-range nature of this signaling and the inability of the mesenchyme to support morphogenesis in the absence of PTHrP or the PPR1 all support this model. PTHrP has been shown to participate in fetal bone morphogenesis in part by directly regulating the differentiation of proliferating chondrocytes within the growth plate (Chung et al., 1998). In addition, PTHrP has been shown to regulate the morphogenesis of several epithelial organs other than the mammary gland, such as tooth, hair follicles and lung and, at these sites, it most likely also contributes to the regulation of epithelial-mesenchymal interactions (Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994). The current data suggest that PTHrP regulates epithelial morphogenesis in the fetal mammary gland by regulating mesenchymal cell fate decisions and we anticipate that this will be the case in other organs as well.

The authors are indebted to Drs Andrew Karaplis, Beate Lanske and Henry Kronenberg for graciously sharing PTHrP- and PPR1-knockout mice. We thank Drs Kaechoong Lee and Gino Segre for sharing unpublished data. We thank Drs Arthur Broadus, William Philbrick and David Stern for helpful discussions during the preparation of the manuscript. Finally, we are grateful for the use of the microscopy facilities of the Cell Biology Core of the Yale Diabetes and Endocrine Research Center. This work was supported by NIH grant CA60498, DOD grant DAMD17-96-1-6198 and a pilot project grant from the Yale Diabetes and Endocrine Research Center (NIH 5-P30-DK45735). M. E. D. is supported by the DOD postdoctoral fellowship DAMD17-97-1-7137.

REFERENCES

Behrendtsen, O., Alexander, C. M. and Werb, Z. (1995). Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos. *Development* **121**, 4137-4146.

Birchmeier, C. and Birchmeier, W. (1993). Molecular aspects of mesenchymal-epithelial interactions. *Ann. Rev. Cell Biol.* **9**, 511-540.

Chen, F. and Capecchi, M. D. (1999). Paralogous mouse Hox genes Hoxa 9, Hoxb 9, and Hoxd 9, function together to control development of the mammary gland in response to pregnancy. *Proc. Natl Acad. Sci. USA* **96**, 541-546.

Chung, V., Lanske, B., Lee, K., Li, E. and Kronenberg, H. (1998). The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc. Natl Acad. Sci. USA* **95**, 13030-13035.

Cunha, G. R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74**, 1030-1044.

Cunha, G. R., Young, P., Christov, K., Guzman, R., Nandi, J., Talamsutes, F. and Thorderson, G. (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* 152, 195-204.

Cunha, G. R. and Hom, Y. K. (1996). Role of mesenchymal-epithelial interactions in mammary gland development. *J. Mamm. Gland Biol. Neopl.* **1**, 21-35.

de Stolpe, A., Karperian, M., Lowik, C. W. G. M., Jüppner, H., Segre, G. V., Abou-Samra, A. B., deLaat, S. W. and Defize, L. H. K. (1993). Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell Biol.* **120**, 235-243.

Dunbar, M. E., Young, P., Zhang, J. P., McCaughern-Carucci, J., Lanske, B., Orloff, J. J., Karaplis, A., Cunha, G. and Wysolmerski, J. J. (1998). Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein. *Dev. Biol.* **203**, 75-89.

Erickson, H. P. and Bourdon M. A. (1989). Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annual Rev. Cell Biol.* **5**, 71-92.

Gilbert, S. F. (1994). Sex determination. In *Developmental Biology* pp. 754-787. Sunderland, MA: Sinauer Associates.

Grumbach, M. M. and Conte, F. A. (1992). Disorders of sex differentiation.

In *Williams Textbook of Endocrinology 8th Edition.* (ed. J. D. Wilson and D. W. Foster) pp. 853-952. Philadelphia, PA: W. B. Saunders Co.

Heuberger, B., Fitzka, I., Wasner, G. and Kratochwil, K. (1982). Induction of androgen receptor formation by epithelial-mesenchymal interaction in embryonic mouse mammary gland. *Proc. Natl Acad. Sci. USA* **79**, 2957-2961.

Hoshino, K. (1965). Development and function of mammary glands of mice prenatally exposed to testosterone propionate. *Endocrinology* **76**, 789-794.

Inaguma, Y., Kusakabe, M., Mackie, E. J., Pearson, C. A., Chiquet-Ehrisman, R. and Sakakura, T. (1988). Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev. Biol.* **128**, 245-255.

Jüppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Hock, J., Potts, J. T., Kronenberg, H. M. and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254**, 1024-1026.

Kalembey, I., Yoshida, T., Iriyama, K. and Sakakura, T. (1997). Analysis of tenascin mRNA expression in the murine mammary gland from embryogenesis to carcinogenesis: an in situ hybridization study. *Int. J. Dev. Biol.* **41**, 569-573.

Kopan, R. and Fuchs, E. (1989). A new look at an old problem: keratins as tools to investigate determination morphogenesis and differentiation in skin. *Genes Dev.* **3**, 1-15.

Kratochwil, K. (1977). Development and loss of androgen responsiveness in the embryonic mammary rudiment of the mouse mammary gland. *Dev. Biol.* **61**, 358-365.

Kratochwil, K and Schwartz, P. (1977). Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue for testosterone. *Proc. Natl Acad. Sci. USA* **73**, 4041-4044.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J. and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**,1382-1394.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinol.* **136**, 453-463.

Majdic, G., Millar, M. R. and Saunders, P. T. K. (1995). Immunolocalization of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *J Endocrinol.* **147**,285-293.

Neumann, F., Berswordt-Wallrabe, Elger, W., Steinbech, H., Hahn, J. D. and Kramer, M. (1970). Aspects of androgen-dependent events as studied by anti-androgens. *Recent Prog. Horm. Res.* **26**, 337-410.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E. and Stewart, A. F. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol. Rev.* **76**, 127-173.

Philbrick, W. M., Dreyer, B. E., Nakchbandi, I. A. and Karaplis A. C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc. Natl Acad. Sci. USA* **95**, 11846-11851.

Phippard, D. J., Weber-Hall, S. J., Sharpe P. T., Naylor, H., Jayatalake, H., Mass, R., Woo, D., Roberts-Clark, D., Francis-West, P. H., Liu, Y. H., Maxson, R., Hill, R. E. and Dale, T. C. (1996). Regulation of Msx-1, Msx-2, Bmp-2 and Bmp-4 during foetal and postnatâl mammary gland development. *Development* **122**, 2729-2737.

Propper, A. and Gomot, L. (1967). Interactions tissulaires au cours de l'organogenèse de la gland mammaire de l'embryon de Lapin. *C. R. Acad. Sci. Paris* **264**, 2573-2575.

Raynaud, A. (1949). Nouvelles observations sur l'appareil mammaire des foetus de souris provent de mères ayant reçu des injections de testostérone pendant la gestation. *Annals Endocrin.* **10**, 54-62.

Raynaud, A. and Frilley, M. (1947). Destruction des glandes genitales de l'embryon de souris per une irradiation au moyen des rayon x, a l'aye de treizejours. *Ann. Endocrinol. Paris* **8**, 400-419.

Robinson, G. W., Karpf, A. B. C. and Kratochwil, K. (1999). Regulation of mammary gland development by tissue interaction. *J. Mamm. Gland Biol. Neopl.* **4**,

Rubin, L. P., Kifor, O., Hua, J., Brown, E. M. and Torday, J. S. (1994). Parathyroid hormone (PTH) and PTH-related protein stimulate surfactant phospholipid synthesis in rat fetal lung, apparently by a mesenchymal-epithelial mechanism. *Biochim. Biophys. Acta* **1223**, 91-100.

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992). Mice develop normally without tenascin. *Genes Dev.* **6**, 1821-1831.

Salmivirta, M., Elenius, K., Vaino, S., Hofen, V., Chiquet-Ehrisman, R., Thesleff, I. and Jalkanen, M. (1991). Syndecan from embryonic tooth mesenchyme binds tenascin. *J. Biol. Chem.* **266**, 7733-7739.

Sakakura, T. (1987). Mammary embryogenesis. In *The Mammary Gland, Development, Regulation and Function,* (ed. M. C. Neville, C. W. Daniel) pp 37-65. New York: Plenum Press.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39**, 35-50.

Turner, C. W. and Gomez, E. T. (1933). The normal development of the mammary gland of the male and female albino mouse. I. Intra-uterine. *Bull. Mo. Agric. Exp. Stn* **182**, 3-20.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R., Parslow, T. G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8**, 2691-2703.

Weil, M., Itin, A. and Keshet, E. (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121**, 2419-2428.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M. and Philbrick, W. M. (1994). Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl Acad. Sci. USA* **91**, 1133-1137.

Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E. and Philbrick, W. M. (1995). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* **121**, 3539-3547.

Wysolmerski, J. J. and Stewart, A. F. (1998). The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. *Ann. Rev. Physiol.* **60**, 431-460.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., Karaplis, A. and Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**, 1285-1294.